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Effect of Furacin (5-Nitro-2-Furaldehyde Semicarbazone) on the *in vitro* Metabolism of Mammalian Tissues.* (19444)

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The effect of the antimicrobial nitrofuran, Furacin(1), on various bacterial enzyme systems has recently been described by Green (2,3), and by Asnis and Gots(4,5). Since the nitrofurans are being studied as systemic agents in the treatment of various pathological conditions, it is essential to determine the effect of such chemotherapeutic agents on the host as well as on the invading organism. Chronic oral administration of nitrofurans to laboratory animals has been associated with few histological changes other than reversible effects on seminiferous tubules and adrenal cortex(6-8). The most prominent symptom following single massive oral doses of Furacin to animals is hyper-irritability with occasional convulsions(6,8).

Studies of the effects of Furacin on tissue metabolism *in vitro* have been initiated and certain of the observed data are presented in this paper. These studies should aid in explaining some of the observed effects of Furacin in the mammalian organism (hyper-irritability, etc.). Combined with such studies as those of Green(2,3), or of Asnis and Gots(4,5), any difference in action of Furacin on mammalian and bacterial cells might be elucidated.

Experimental. Measurements of oxygen uptake were made with standard Warburg apparatus in an atmosphere of oxygen in Krebs-Ringer-phosphate buffer(9) pH 7.2. Anaero-

* This paper was presented in part before the American Chemical Society, Boston, April 1951.

TABLE I. Effect of Furacin on Oxygen Uptake of Various Rat Tissues Under Endogenous Conditions and with Added Glucose.

Tissue	Substrate	Furacin conc.	No. flasks	Qo_2* 30 min	Qo_2* 1 hr	Qo_2* 2 hr
Liver	Endogenous	—	3	3.2	3	2.3
	”	‡	3	2.9	2.7	1.3
	Glucose .2%	—	5	3.9	3.5	2.6
	” ”	‡	6	4.8	3.2	1.8
Kidney	Endogenous	—	6	16.1	14.5	12.3
	”	‡	5	16.7	13.2	10.3
	Glucose .2%	—	3	16.4	16	14.3
	” ”	‡	3	19.7	17.9	15.2
Brain	Endogenous	—	6	4.8	3.3	1.5
	”	‡	6	5.4	2.5	.6
	Glucose .2%	—	3	11.1	10.1	9.2
	” ”	‡	3	11.9	8.3	2.7
Diaphragm	Endogenous	—	3	4.5	3.4	3
	”	‡	3	4.5	1.8	.7
	Glucose .2%	—	3	5.4	4.4	3.1†
	” ”	‡	3	5.8	3.3	1 †
Testis	Endogenous	—	3	2.9	2.5	1.1
	”	‡	3	2.4	.8	.4
	Glucose .2%	—	3	6.2	5	3.3
	” ”	‡	3	4.5	3.1	2.2
Heart	Glucose .2%	—	6	2.8	2.7	1.8
	” ”	‡	6	2.1	1.5	.6

* mm^3 O_2/mg dry wt tissue/hr. † Value at 1 hr 45 min. Exp. not continued for 2 hr.
‡ Furacin conc. 7×10^{-4} M.

bic studies were made in bicarbonate buffer (9) in an atmosphere of 95% N_2, 5% CO_2. Tissue slices of rat liver, heart, kidney cortex and brain and of guinea pig kidney cortex and brain were prepared with the Stadie slicer (10) with the usual precautions. Rat testis preparations were made by teasing apart the tubules. Rat diaphragm muscle was used as strips. Furacin crystals were dissolved in the buffer-substrate medium, the concentration adjusted to the desired level after spectrophotometric determination(11), and this drug-containing medium introduced into the experimental flasks at the start of the experiment. Control flasks containing the medium without added drug were set up concurrently for comparison in all cases. Tissues and flask contents were chilled until all were prepared to facilitate simultaneous transfer to the Warburg bath. Substrate concentration is recorded in the individual graph or table for each experiment. Readings were taken at 15-minute intervals and the results calculated as the appropriate Q values for the interval over a 1½- to 3-hour period.

Results. The effect of Furacin upon the endogenous metabolism of the selected tissues and upon the metabolism of the same tissues in the presence of added glucose is shown in Table I. It may be seen that there is relatively little difference between the endogenous metabolism of liver and kidney and the metabolism in the presence of added glucose, indicating presence of endogenous substrate in these tissues. A carry-over of substrate by diaphragm is also indicated. The dependence of brain and testis on added substrate is shown by the rapid decrease in Qo_2 in the endogenous study and the maintenance of Qo_2 in the presence of added glucose. Marked interference in glucose metabolism of brain and testis and some interference with that of heart and diaphragm is noted in the presence of Furacin. Little or no interference by Furacin with the oxygen uptake by kidney or liver slices, either endogenous or in the presence of added glucose, is observed.

Of the tissues used, brain slices appeared the most suitable for more extended studies for the following reasons: (a) the endogenous studies confirm the observations of previous workers(12) that relatively little stored substrate is present in this tissue. This simplifies the picture when various substrates are to be

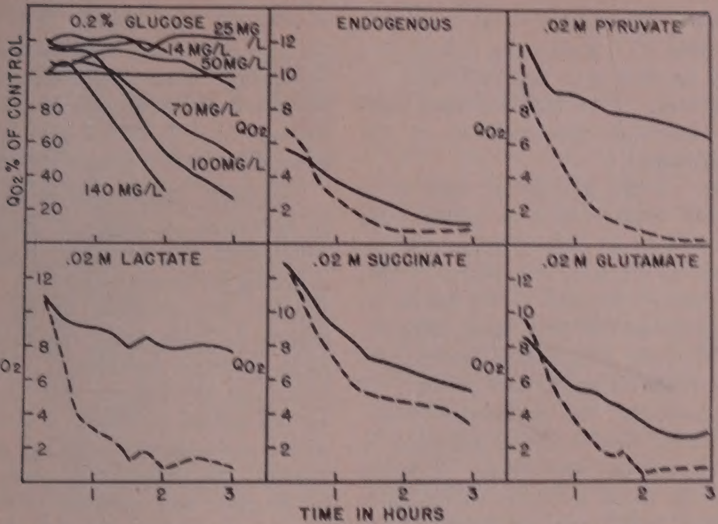

FIG. 1. Effect of Furacin on oxygen uptake of rat brain slices in various substrates. Furacin concentration = 140 mg per liter (7×10^{-4}M) except as noted in the glucose substrate. ——— control; - - - - - Furacin.

studied. (b) the Q_{O_2} is high and differences due to inhibition show up readily. (c) it was of interest to continue *in vitro* studies on brain since hyperirritability and convulsions were the most obvious symptoms observed after high oral doses of Furacin.

The results of studies on the effect of various concentrations of Furacin on the metabolism of brain slices, using glucose as a substrate, are presented in Fig. 1. In this portion of the graph, the Q_{O_2} values are presented in terms of per cent of control values rather than as absolute figures in order to compare directly the results from different experiments. Here it can be seen that the inhibition of oxygen uptake by Furacin decreases with decreasing concentration of the drug. At the lower levels no inhibition occurs under the conditions of the experiment. The increase in Q_{O_2} observed at the lower concentrations of Furacin and during the initial periods of the higher concentrations cannot be explained at this time. Similar effects have been observed with methadone • HCl (Amidone) (13) and with sodium salicylate(14).

In attempting to locate more definitely the site of action of Furacin on carbohydrate metabolism, the effect of the drug on the respiration of brain slices in the presence of various substrates was studied. The results are shown in Fig. 1. The inhibiting effect of Furacin is most apparent in the metabolism of pyruvate and of lactate and least marked in the presence of succinate. The interference with pyruvate metabolism has also been corroborated by analytical keto-acid determinations. The following substrates were also used but did not appear to increase the respiration of rat brain slices above the endogenous level: malate, fumarate, citrate, acetate, aspartate, and glycine.

The effect of Furacin on the oxygen uptake of rat kidney cortex slices was determined under endogenous conditions and using glucose, pyruvate, lactate, succinate, glutamate, aspartate, citrate, and acetate as substrates. In the order of substrates named the Q_{O_2} values at the end of the 1-hour period were as follows: 14.5 (endog.), 16.2, 18.4, 22.6, 30.4, 25.4, 20.9, 14.5, 20.5. The only medium in which significant Furacin inhibition occurred was pyruvate (Q_{O_2} at 1 hr. = 75% of control and 60% at 2 hr).

Anaerobic glycolysis of glucose as measured by CO_2 production from bicarbonate buffer was studied, using rat brain and testis and guinea pig brain and kidney cortex slices. The control $Q_{CO_2}^{N_2}$ values observed at one hour

were as follows: rat brain 6.7, rat testis 6.3, guinea pig brain 10.7, guinea pig kidney cortex 5.4. In no case was any inhibition by Furacin observed. This has also been confirmed by analytical determination of the lactic acid produced.

Discussion. When glucose is used as a substrate with brain tissue, a rather long latent period (Fig. 1) occurs before the inhibitory effect of Furacin is apparent. This probably cannot be ascribed to the failure of Furacin to penetrate cell walls since, with the same type of tissue preparation (slices), no such latent period occurs in the presence of the other substrates. No appreciable interference by Furacin with anaerobic glycolysis in mammalian tissues has been found. It seems possible that the latent period in the presence of glucose may result from temporary regeneration, during glycolysis, of accessory factors necessary for certain limited oxidative pathways which may operate in the presence of Furacin. Balance studies using brain tissue with glucose as a substrate are in progress in this laboratory and the results of determinations of glucose disappearance and end-product appearance in the presence and absence of Furacin should throw further light on the mechanism of Furacin action.

The absence of inhibition in anaerobic glycolysis and the effects on aerobic metabolism in the presence of various substrates suggest that the mechanism sensitive to Furacin inhibition in the mammalian cell occurs in the aerobic utilization of pyruvate. The studies of Asnis and Gots(4) and of Green(2) on bacterial cells indicate interference by Furacin in the enzymatic dehydrogenation systems of bacteria involved in carbohydrate metabolism in both aerobic and anaerobic systems.

The present metabolic studies indicate that mammalian tissues (brain, testis) which have shown gross manifestations or histological evidence of effects from high doses of Furacin *in vivo* are tissues which are dependent on carbohydrate substrates for maintenance of *in vitro* metabolism. Interference by Furacin with the glucose metabolism of these tissues *in vitro* has been shown. It is suggested that the hyperirritability and convulsions produced by massive doses of Furacin as well as the changes in the testes may be related in a large measure to a depressed aerobic carbohydrate metabolism in the presence of the nitrofuran.

Summary. Investigations were carried out to determine the effect of Furacin upon the metabolism of mammalian tissue slices using Warburg manometric technics. Inhibition by Furacin was observed in the utilization of glucose by brain, testis, diaphragm, and heart under aerobic conditions. No appreciable inhibition by Furacin was observed in the oxygen uptake of kidney or liver slices either under endogenous conditions or with added glucose. No inhibition of anaerobic glycolysis by Furacin was observed in brain, testis or kidney. Studies on the aerobic utilization of various substrates by brain and kidney slices point to the inhibition by Furacin as occurring in the aerobic utilization of pyruvate.

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Decreased Resistance of Riboflavin-Deficient Rats to Cold Stress.* (19445)

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Considerable data are available indicating that resistance to cold stress may be significantly impaired in the nutritionally-deficient animal. Thus an impaired resistance to cold has been demonstrated in rats deficient in vit. A(1,2) and pyridoxine(3) and guinea pigs deficient in vit. C(4). In the present communication data are presented indicating that riboflavin deficiency also impairs adjustment to low environmental temperature in the rat.

Procedure and results. Five experimental rations were employed in the present investigation: diets A, B, C, D, and E. Diet A was a riboflavin-free ration of the following composition: sucrose, 60%; casein,† 25%; salt mixture,‡ 5%; cottonseed oil (Wesson), 10%; and the following synthetic vitamins per kg of diet: thiamine hydrochloride, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; ascorbic acid, 200 mg; biotin, 5 mg; folic acid, 10 mg; *p*-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12}, 150 μg; 2-methylnaphthoquinone, 10 mg; and choline chloride, 2 g. To each kg of diet were also added 4000 U.S.P. units of vit. A§ and 400 U.S.P. units of vit. D.‖ The vitamins were added in place of an equal amount of sucrose. Each rat also received once weekly a supplement of 4.5 mg alpha-tocopherol acetate. Diets B, C, D, and E were similar in composition to diet A, but contained in addition the following amounts of riboflavin per kg of diet: diet B, 0.5 mg; diet C, 1.5 mg; diet D, 10 mg; and diet E, 50 mg. One hundred and eight female rats of the Long-Evans strain were selected for the present experiment at 23 to 28 days of age and an average weight of 58.4 g. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad libitum* (diet A was fed to 28 rats; diet B to 22; the remaining groups consisted of 20 rats each). Diets were made up weekly and stored under refrigeration when not in use. Animals were fed on alternate days. All food not consumed 48 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet. After 3 weeks of feeding the following average body weights were obtained for rats in the various dietary groups: diet A, 71.2 g; diet B, 115.8 g; diet C, 118 g; diet D, 143.2 g; and diet E, 144.5 g. At this time 25 of the 28 rats on diet A were depleted of riboflavin as judged by stationary or decreasing body weight for a period of 7 days; all rats on diets B, C, D, and E were gaining weight. Animals in the various dietary groups were then divided into 2 series. Ten rats in each group were continued at standard laboratory conditions (23°C); the remaining animals in each group were placed in a walk-in refrigerator maintained at a temperature of 2 ± 1.5°C. No change was made in the diets fed. Feeding was continued *ad libitum* for an additional 8 weeks or until death, whichever occurred sooner. Results are summarized in Table I.

Findings indicate that rats depleted of riboflavin failed to survive under conditions of low environmental temperature. One hundred per cent of the rats fed diet A died under cold room conditions with an average survival time of 7.6 days (range 2 hours to 17 days). Under room temperature conditions, however, 60% of the rats fed a similar ration were still

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† Vitamin Test Casein, General Biochemicals, Chagrin Falls, O.

‡ Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

§ Myva-Dry Powder, Distillation Products, Rochester, N. Y.

‖ HY-Dee Powder, Standard Brands, New York.

TABLE I. Effects of Graded Doses of Riboflavin on Growth and Survival of Rats Maintained Under Cold Room and Room Temperature Conditions.

Dietary groups	Riboflavin in ration, mg/kg of diet	No. of animals	Initial body wt, g	Change in body wt after 8 wk exp., g	% surviving*	Avg survival time of decedents, days
			Cold room series			
A	0	16	71.6	—	0	7.6
B	.5	12	116.6	−5 (3)	25	22
C	1.5	10	120.9	35 (8)	80	22
D	10	10	146.6	33.9 (10)	100	
E	50	10	144.1	32.4 (10)	100	
			Room temp. series			
A	0	10	70.5	−13.8 (6)	60	43
B	.5	10	114.9	7.5 (10)	100	
C	1.5	10	115.1	57.2 (10)	100	
D	10	10	139.7	57.3 (10)	100	
E	50	10	141.8	66.2	100	

Values in parentheses indicate number of animals surviving on which averages are based.
* Experimental period—56 days.

alive 8 weeks after depletion. A dietary intake of 0.5 mg riboflavin per kg of ration was insufficient to maintain survival under conditions of low environmental temperature. Seventy-five per cent of the rats fed this diet succumbed under cold room conditions with an average survival time of 22 days (range 15 to 33 days). At room temperature conditions, however, this intake of riboflavin was sufficient to keep all rats alive and to promote a slight increment in body weight. Higher levels of riboflavin (1.5, 10 and 50 mg per kg of ration) permitted growth and survival both under cold room and room temperature conditions.

Discussion. It is well established that requirements for adrenal cortical hormones are markedly increased under conditions of low environmental temperature(5-7) and that adrenalectomized or hypophysectomized rats fail to survive following prolonged exposure to cold. Adjustment to cold is also impaired by thyroidectomy(8) or administration of thiouracil(9). Resistance to cold may be restored in adrenalectomized rats by administration of adrenal cortical hormone(s) and in thyroidectomized or thiouracil-fed rats by injection of thyroxin. Present findings indicate that resistance to low environmental temperature (as judged by length of survival) was significantly impaired in rats fed rations containing 0.5 mg of riboflavin per kg of diet or less. Since considerable impairment of adrenal cortical function may occur in the riboflavin-deficient rat(10), further studies are indicated to determine to what extent adrenal cortical (and possibly pituitary and thyroid) dysfunction may have contributed to the decreased resistance of riboflavin-deficient rats noted above. Preliminary findings indicate that neither cortisone acetate¶ (administered at a level of 2.5 mg per day intraperitoneally) nor thyroxin (administered at a level of 30 γ per day intraperitoneally) were effective in prolonging the survival of riboflavin-deficient rats under conditions of low environmental temperature.

Results of the present experiment indicate that a state of nutriture which may be adequate under standard laboratory conditions may be inadequate under conditions of cold stress. Thus riboflavin when administered at a level of 0.5 mg per kg of diet was insufficient to maintain life and body weight under conditions of low environmental temperature, whereas this level was sufficient to keep all rats alive and to promote a slight increment in body weight under room temperature conditions. It is possible, since food intake was reduced in the riboflavin-deficient rats, that the impaired adjustment of riboflavin-deficient rats to cold stress was due, at least in part, not to riboflavin malnutriture *per se* but the attendant reduction in caloric intake. Further experiments are indicated to deter-

¶ The cortisone acetate was kindly provided by Dr. R. A. Peterman of Merck and Co., Rahway, N. J.

mine to what extent this factor may have contributed to the observed results.

Summary. Experiments were conducted on the effects of graded doses of riboflavin on the growth and survival of rats maintained under cold room and room temperature conditions. Weanling rats were fed for 3 weeks on diets containing 0, 0.5, 1.5, 10, and 50 mg of riboflavin per kg of diet. After 3 weeks of feeding (at which time rats on the riboflavin-free diet had plateaued in weight) animals were divided into 2 groups. One group was placed in a cold room maintained at 2°C; the other was maintained at room temperature (23°C). Adjustment to cold (as measured by length of survival) was significantly impaired in rats fed diets containing 0.5 mg of riboflavin per kg of diet or less.

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Presence of Bacteria in the Spleen of Pteroylglutamic Acid Depleted Rats. (19446)

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Gross lesions, infarct like in nature, were reported by Asenjo(1) to develop in the spleens of a considerable number of pteroylglutamic acid (PGA) depleted rats receiving a basal PGA deficient diet containing either succinylsulfathiazole (SST) or x-methyl-folic acid as inhibitor. Later Philips and Thiersch (2) studied the action of 4 amino-pteroylglutamic acid on rats and observed, in 5% of the animals receiving this compound by chronic administration, the appearance of intestinal lesions through which an ascending Salmonella infection took place. As a result of this bacterial invasion enlargement of the mesenteric lymph nodes, abscess formation, multiple fibrinoid necrosis in liver, spleen, kidney, and lung, bronchitis and bronchopneumonia occurred.

The above findings have led us to search for bacteria in the gross splenic lesions obtained in PGA depleted rats receiving SST as inhibitor.

Experimental. Thirteen 21-day-old Wistar albino rats from our colony were kept in cages with raised wire floors. The basal purified diet free of PGA, fed *ad libitum*, had the following composition per kg: sucrose, 599 g; vitamin-free casein, 180 g; salt mixture, 40 g; cellu flour, 40 g; hydrogenated vegetable oil, 100 g; corn oil, 20 g (containing 52,000 I.U. vit. A, 13,000 I.U. vit. D, and 32 mg α-tocopherol); succinylsulfathiazole, 20 g; choline chloride, 1 g; inositol, 8 mg; nicotinic acid, 40 mg; calcium pantothenate, 44 mg; thiamine hydrochloride, 8 mg; riboflavin, 16 mg; pyridoxine hydrochloride, 8 mg; p-aminobenzoic acid, 4 mg; biotin,† 0.5 mg; 2-methyl-1,4-naphthoquinone, 10 mg. The depletion period ranged from 3 to 4 weeks. By the end of the fourth week the majority of the animals

* Project sponsored by the Agric. Exp. Station of the University of Puerto Rico.

† Biotin supplied by Hoffmann-La Roche, courtesy of Dr. E. Sevringhaus.

had reached a weight plateau and a white cell count of 3,000 to 4,000 cells per mm^3. Some of them received from that time until they died, or were sacrificed, suboptimal levels of PGA (not over 0.5 μg PGA per day) others were left on the basal diet only. Those animals that died were autopsied within an hour after death. The large majority of the animals, however, were sacrificed after they were for 8 weeks on the basal diet, by injecting air into the heart. The spleens were removed under aseptic conditions. In addition, 6 infarcted spleens preserved in museum fluid were also examined by dissecting out the necrotic areas and preparing smears from them.

Results. Of the 13 rats depleted of PGA, 3 showed severe gross lesions in the spleen, 4 had spleens with doubtful lesions, and 6 had apparently normal spleens. All the spleens were small in size.

Gram stains of the necrotic areas of the infarcted spleens showed either a large number of gram positive or gram negative cocci and diplococci or slightly pleomorphic diphtheroid gram positive rods. Only one of the doubtful spleens showed bacteria in the direct smear, a small number of gram positive bacilli. In the rest of the doubtful, as well as in all the normal spleens, the smears were negative for bacteria.

Blood agar and thioglycollate broth cultures from the infarcted spleens developed abundant growth of irregularly staining pleomorphic diphtheroid forms, gram positive or gram negative cocci or gram negative coli-like rods. One of the gram negative bacilli was *Escherichia coli,* the others were not identified.

Two of the doubtful spleens yielded a scanty growth of bacteria. In one case, a non-hemolytic group D streptococci, and in the other, of an organism belonging to the proteus group.

Cultures of material from the apparently normal spleens from PGA deficient rats developed only in two instances scanty growth of bacteria: a gram negative unidentified bacillus of coliform morphology, and a slightly pigmented *Micrococcus aureus.*

Direct smears made by dissecting out the necrotic areas of 6 infarcted spleens preserved in museum fluid further confirmed the presence of massive amounts of bacteria in the necrotic tissue. These areas were found to be loaded with pleomorphic diphtheroid bacilli, gram negative bacilli of coli-like morphology or gram positive cocci.

It is interesting to point out that in a series of 541 PGA depleted rats examined post mortem by one of us(3), the incidence of gross splenic lesions was 48%, of gross liver lesions 18%, and of gross lesions in other organs 9%. An almost general finding in these animals was a yellowish distended intestine. In 227 animals in which the tongue was examined, 45% showed the typical lesion observed in PGA deficient rats by Franklin *et al.*(4).

Apparently some of the rats studied suffered from an endogenous infection in which the mouth and intestine probably served as the main portal of entry. This conclusion is based on the fact that the bacteria which were encountered in the splenic lesions were of the types usually found in the alimentary canal.

Summary and conclusions. The necrotic areas in the splenic lesions observed in PGA deficient rats receiving orally SST as inhibitor, were largely made up of bacteria of the type found mainly in the alimentary canal, which suggest that the mouth and intestine were probably the main portal of entry for these organisms.

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Pyrazinoic Acid Amide—An Agent Active Against Experimental Murine Tuberculosis. (19447)

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(With the technical assistance of T. H. Wombolt.)

From Merck Institute for Therapeutic Research and Research Laboratories, Merck and Co., Rahway, N. J.

The antituberculosis efficacy of niacinamide was first described by Chorine(1). He found that doses of 500 to 1000 mg per kg administered subcutaneously produced retardation of experimental tuberculosis in guinea pigs. This effect was later rediscovered by McKenzie *et al.*(2), who reported that a concentration of 0.75% niacinamide in diet prevented the progress of tuberculosis infection in mice. Kushner *et al.*(3) then studied the activity of a series of 30 derivatives covering substitution in the acid amide group and the ring structure. A number of amide nitrogen substituted derivatives were found active but inferior to niacinamide. Isosteres of niacinamide were not mentioned in this report. Studies by Fox(4) also confirmed the *in vivo* activity of niacinamide and added additional compounds to the series of inactive niacinamide-like compounds. At the Eleventh Conference on Chemotherapy of Tuberculosis, sponsored by the Veterans Administration, St. Louis, Jan. 17, 1952, S. Kushner of Lederle, R. L. Yeager, W. G. C. Munroe and F. I. Desseau from Summit Park Hospital and W. S. Schwartz from Oteen, a facility of the Veterans Administration, announced that pyrazinoic acid amide (Aldinamide) was active against experimental tuberculosis in the mouse and against clinical infection in man (5).

Studying niacinamide isosteres, we have likewise found that pyrazinoic acid amide is more active than niacinamide or para aminosalicylic acid when administered as components in diet.

Materials and Methods. The H37Rv strain of *Mycobacterium tuberculosis*, human type, was used in these studies. It was maintained by weekly serial passage in Dubos' Tween-Albumin medium containing 0.03% Tween 80 and virulence of strain was preserved by passage through mice after five transfers in culture medium. Barckmann IS-32 white mice weighing 14 to 19 g were injected intravenously with 0.25 ml of culture suspension adjusted with sterile distilled water to permit 70% transmission of light at 620 mμ.* Treatment was begun on the day following infection. For subcutaneous administration, test compounds were dissolved in sterile distilled water at concentrations containing daily doses per mouse in 0.25 ml of solution. Compounds given by subcutaneous route were administered once daily 5 days per week. For administration in diet the compounds were ground and incorporated into a stock diet† presented *ad libitum*. Experiments were continued until at least 50% of infected untreated animals died. Mice dying during the course of the experiment as well as those surviving at the end of test period were autopsied and lungs were preserved in 10% formalin. After a minimum of 48 hours of fixation the lungs were observed for the extent of gross tuberculosis involvement. The degree of involvement was graded as 0 for no visible involvement, 1+ (to 10% of lung tissue, grossly involved), 2+ (10 to 25% gross involvement) 3+ (25 to 50% gross involvement) and 4+ (more than 50%

* On a Coleman photoelectric spectrophotometer, Model 6A.

	%
† Yellow corn meal	31
Whole wheat flour	30
Casein, technical	10
Soy bean meal	10
Linseed oil meal	7
Yeast dried brewers	5
Alfalfa meal	2
NaCl	0.5
$CaCO_3$	0.5
Mazola oil	3
Cod liver oil	1
10 mg. vit. B_2/kg.	

involvement). Extensive experience has shown that gross scores correspond closely with values obtained by histological examination. *In vitro* efficacy tests were performed both with Dubos' Tween-albumin medium and Youmans' modification of Proskauer and Beck medium. Concentrated solutions of pyrazinoic acid amide were sterilized by filtration through Seitz EK pads and added aseptically to the test medium to yield final concentrations to 1000 γ/ml. The tubes were inoculated with 0.1 ml of culture in Dubos' medium adjusted to permit 90% transmission of light at 620 mμ and inspected for growth after 14 days of incubation at 37°C. For studying development of resistance, standardized culture suspension was added to a series of tubes containing 40 to 400 γ of pyrazinoic acid amide per ml of medium. After 2 weeks incubation, culture from the tube with a turbidity of approximately 90% transmission was transferred to a fresh series of tubes containing higher concentrations of compound. This procedure was continued at 2-week intervals.

Experimental. 1) *Comparison of pyrazinoic acid amide with niacinamide.* In a typical experiment to determine efficacy in diet, the compounds were tested at 0.1, 0.5 and 1.0% concentrations using groups of 8 mice each. Pyrazinoic acid amide at 1 and 0.5% concentration gave a score of .0 and at .1% the score was 0. Niacinamide gave a score of 1.4 at 1%, 2.5 at 0.5% and 3.7 at 0.1%. Thus pyrazinoic acid amide produced complete suppression of visible tuberculosis lesions at levels where niacinamide treated mice showed minimal to moderate involvement. The compounds did not significantly influence acceptability of the diet and the weights of mice treated with effective levels of compound were similar to those of normal controls. *Pyrazinoic acid amide* was also superior to niacinamide when the compounds were administered by subcutaneous injection. The compounds were tested at doses of 20, 10 and 5 mg per day administered 5 days per week. Both compounds gave scores of less than 0.5 at 20 mg. At the 10 mg level, pyrazinoic acid amide continued to give a score of less than 0.5 but niacinamide gave a score of 1.2. At a level of 5 mg niacinamide was ineffective but pyrazinoic acid amide gave a score of 2.

TABLE I. Comparison of Pyrazinoic Acid Amide with Para-Aminosalicylic Acid, Administration by Subcutaneous Route.

Dose, mg/mouse/day	Lung score pyrazinoic acid amide	Para-amino-salicylic acid
Trial 1. Infection of high virulence (avg survival time, 20 days)		
1	3.3	3.2
2	1.5	.9
3	1.6	1
5	.6	.4
Lung score of infected controls—3.7		
Trial 2. Infection of low virulence (avg survival time, 33 days)		
1.25	4	4
2	3.6	4
3	3.1	3.9
4	1.1	4
Lung score of infected controls—3.6		

2) Comparison of pyrazinoic acid amide with para-aminosalicylic acid. Pyrazinoic acid amide and para-aminosalicylic acid were compared for efficacy as components in diet and by parenteral administration.

A) For comparison by parenteral route 2 trials are presented in Table I. In the first, pyrazinoic acid amide and para-aminosalicylic acid were administered subcutaneously in doses of 1, 2, 3, and 5 mg per mouse per day. Groups of 8 mice each were used. Both compounds demonstrated slight efficacy at 2 and 3 mg and good efficacy at 5 mg. On actual score basis, para-aminosalicylic acid showed slightly higher activity than pyrazinoic acid amide but the difference, approximately 20%, does not appear highly significant. In a second trial using similar doses and groups of eight mice, but testing with a culture of lower virulence, pyrazinoic acid amide was superior to para-aminosalicylic acid. Para-aminosalicylic acid was ineffective at all levels up to 4 mg per mouse per day whereas pyrazinoic acid amide showed slight efficacy at doses of 3 mg and good efficacy at 4 mg. The superiority of pyrazinoic acid amide when tested against infection characterized by longer average survival time was also observed when the compounds were administered as components of diet.

TABLE II. Comparison of Pyrazinoic Acid Amide with Para-Aminosalicylic Acid, Administration of Compounds in Diet.

Compound	% in diet	Gross lung scores: Undiluted inoculum	Gross lung scores: 10^{-1} dilution inoculum
Pyrazinoic acid amide	.1	4	3.9
	.5	.1	.3
	1		.1
	2		
Para-amino-salicylic acid	.1	2.6	3.2
	.5	1.2	3.3
	1	.1	1.9
	2		.8
Infected controls		4	4

B) For comparison by diet method a trial is presented in which both drugs were tested in mice infected with standardized suspension of a highly virulent sub-culture of strain H37Rv and a 10-fold dilution of the standardized suspension. Drug diet concentrations of 0.1, 0.5, 1 and 2% were given to groups of 8 mice. The average survival time was 14 days for infected controls inoculated with the undiluted culture suspension and 33 days for infected controls inoculated with diluted culture suspension. The lung scores are shown in Table II. As was observed with administration of the drug by parenteral route, the relative activity of pyrazinoic acid amide as compared with para-aminosalicylic acid when administered in a drug diet mixture was greater in the animals subjected to the slower infection. While a superiority of pyrazinoic acid amide was evident with the undiluted suspension, this compound showed significantly higher activity against infection produced with the diluted culture suspension. Also, the relative efficacy attained with pyrazinoic acid amide increased with rising concentration of drug in the diet.

Pyrazinoic acid amide did not show a high degree of *in vitro* activity. The inhibiting concentration was 150 γ/ml in Dubos' medium and 120 γ/ml in Youmans medium. Resistance studies were performed with Dubos' Tween-Albumin medium. The inhibitory concentration rose from 150 γ/ml to more than 1000 γ/ml after 3 serial transfers.

Conclusions. 1. These data contribute to the importance of niacinamide derivatives as a new class of antituberculosis agents. One of these, pyrazinoic acid amide, is active when administered by parenteral or oral route. Under suitable experimental conditions, pyrazinoic acid amide is more active than para-aminosalicylic acid; the activity of pyrazinoic acid amide as compared with para-aminosalicylic acid increases when the average survival time for infected controls is prolonged. 2. The *in vitro* inhibitory concentration for *M. tuberculosis*, human type, strain H37Rv, tested in Dubos' Tween-Albumin medium, is 150 γ/ml. Resistance develops rapidly *in vitro*; the inhibitory concentration rises to more than 1000 γ/ml after 3 serial bi-weekly transfers.

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Quantitative Distribution of Phosphorus in Chorioallantoic Membrane as Affected by Infection with Influenza Virus.* (19448)

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In recent years interest has been focused on the chemical changes occurring in host tissues as a result of virus infection. Demonstration of chemical changes occurring in the host should make possible a more detailed study on the intermediate mechanisms of infection. This paper is a preliminary report dealing with the quantitative distribution of various phosphorus fractions in the chorioallantoic membrane as affected by infection with the PR-8 strain of influenza virus.

The multiplicity of organs and tissues present in the intact animal or embryonated egg make studies of this nature difficult and equivocal. The de-embryonated egg(1), with its isolated chorioallantoic membrane, was decided upon in an attempt to utilize a relatively more homogeneous cell population. Although this is essentially a tissue culture system, high titers of influenza virus can still be consistently produced in 24 hours, with little gross pathology evident in the membrane.

Material and methods. Eggs of 15 days incubation were de-embryonated and the adherent chorioallantoic membrane carefully washed with four 15 ml rinses of cold isotonic saline. The de-embryonated eggs were then partly filled with 10 ml of Hanks' solution(2), containing 10 units of penicillin and 40 μg of streptomycin per ml. In the virus experiments allantoic fluids from eggs infected with the PR8 strain of influenza A were diluted a 100-fold in the Hanks' solution which was added to each egg. These diluted fluids contained approximately 10 hemagglutinating units per ml. The eggs were capped and rolled (1 rpm) at 37°C. After varying intervals of incubation, the membranes of 2 eggs were removed from the shell, pooled, blotted in a constant fashion, and minced with fine scissors. The mince was then transferred to a tared 15 ml centrifuge tube and weighed on a chainomatic balance. This 2-membrane-pool then served as the material for subsequent analysis. Hemagglutination determinations were made on the suspending fluids at time of harvesting, according to the method of Salk(3). For the measurement of the phosphorus fractions the extraction procedure was the Schmidt and Thannhauser method(4), modified in that small amounts of tissue were used, and tissue residues were centrifuged. Phosphoprotein phosphorus was separated by the Delory(5) procedure. Aliquots of the various fractions were analyzed for total phosphorus by the method of Fiske and Subbarow(6).

Results. Table I includes the averages of the results of 5 experiments designed to show changes in the phosphorus content of the isolated chorioallantoic membrane after 12 and 24 hours incubation, both with and without virus. The slight difference in the nucleic acid fractions between the infected and normal membranes at 24 hours are not significant with the number of experiments completed. The striking decreases in the phosphorus content of the phospholipid fraction of the infected tissue seemed important enough so that further experiments were done at other intervals of infection.

Table II shows the values of phospholipid phosphorus for infected and noninfected membranes. In each case the value given is the average of 5 determinations. A rapid decrease in the phospholipid phosphorus occurred during the first 12 hours of infection. The great variations in the values for the 8-hour determination suggest a rapid flux at this period. Fig. 1 shows the relationship between the decrease in the phospholipid phosphorus and the rise in hemagglutinin titer.

Semi-quantitative determinations were performed on the fluid medium from the infected de-embryonated eggs in an effort to account for the phospholipid which disappeared from the membrane. The medium was dehydrated

* This work was supported in part by grants from the Rockefeller Foundation and the National Institutes of Health, U. S. Public Health Service.

TABLE I. Concentration of Phosphorus Fractions in Infected and Non Infected Chorioallantoic Membranes at 12 and 24 Hr of Incubation.

Fraction	γ P/500 mg tissue 12 hr incubation No virus	Virus	γ P/500 mg tissue 24 hr incubation No virus	Virus
Total acid soluble	820	826	834	821
Lipid phosphorus	320	109	326	120
Total acid insoluble	376	384	380	410
Desoxyribonucleic acid	162	158	160	180
Ribonucleic acid	216	224	220	240
RNA/DNA ratio	1.33	1.41	1.38	1.33

TABLE II. Effect of Virus Multiplication on Phospholipid Phosphorus Content of Infected and Non-Infected Membranes.

Incubation time, hr	Phospholipid concentration, γ P/300 mg tissue Non-infected	Infected
0	327 ± 16.3*	320 ± 19.2
8	324 ± 12.9	191 ± 28.7
12	334 ± 20	110 ± 5.5
16	326 ± 14.7	126 ± 8.82
20	320 ± 18.4	112 ± 6.7
24	325 ± 15.1	120 ± 6
36	309 ± 9.2	114 ± 5.1

* Stand. dev. $= \sqrt{\frac{\Sigma d^2}{N-1}}$.

in vacuo, and phospholipid was extracted according to the Schmidt-Thannhauser(4) method after the initial extraction with aqueous trichloracetic acid. The lipid extract was analyzed for phosphorus. The results indicate that at least 50-60% of the phosphorus liberated by action of the virus in the membrane remains lipid-bound in the medium.

Another group of experiments was performed, in which 5 μc of P^{32} was added per egg. The membranes were minced and extracted in the standard manner, and both P^{31} analysis and P^{32} counts were done on each fraction. There was a marked increase in the specific activity of the phospholipid fraction, as shown in Table III. The RNA values showed increases in activity in the infected group, but this cannot be considered too significant, since small and variable amounts of contamination by phosphoprotein phosphorus would considerably alter the values.

Discussion. With the procedures utilized, the nucleic acid phosphorus concentrations following infection were not significantly altered during the time periods studied. The rapid decreases in phospholipid phosphorus, and the presence of considerable quantities of lipid-bound phosphorus in the culture medium, suggest that phospholipid molecules as such or in combination with other cellular constituents are liberated from a cell infected with the influenza virus. Since not all of the liberated phosphorus could be accounted for in the culture medium as lipid-bound phosphorus, it may be that some is liberated in the form of inorganic phosphorus.

The increased uptake of P^{32} into the phospholipid fraction following infection may be explained either by increased phospholipid synthesis, or possibly as a result of a phosphoroclastic type of reaction.

It would be of utmost importance if one

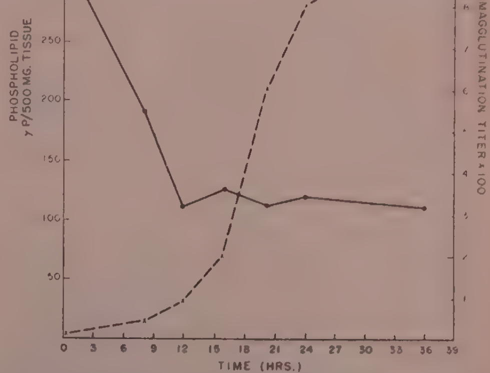

FIG. 1. Relation between decrease in phospholipid content and rise in hemagglutination titer.

TABLE III. Effects of Virus Multiplication on Relative Specific Activity of Phosphorus Fractions at 24 Hr Incubation.

Fraction	Relative specific activity*	
	Non-infected	Infected
Phospholipid	3.15	5.86
Desoxyribonucleic acid	.65	.67
Ribonucleic acid	3.60	4.20

* Rel. spec. activity—Specific activity (counts/minute µg of P^{32}) of each fraction as % of specific activity of inorganic P fraction.

could ascertain the site of the reacting phospholipid in the cell. It has been shown by Friedkin(7) that mitochondria, which contain large amounts of phospholipids, take up P^{32} and incorporate it into their phospholipid fraction. In view of Friedkin's work, it is an interesting consideration that the observed changes in P^{32} uptake of infected tissue may occur in the mitochondria.

Since it is also known that considerable phospholipid is found in cell membranes, there may be a relation between phospholipid liberation and the penetration of the virus particle into the cell. The work of Howe(8) who found large amounts of the red cell receptor in a stroma complex rich in phospholipid, seems pertinent.

Because it has been shown(9) that large differences in the proportion between the hemagglutinating and infectious properties of influenza virus may occur in de-embryonated eggs, nothing can as yet be postulated concerning these findings and the infectious form of the virus.

Summary. It has been shown that infection of the chorioallantoic membrane by the PR-8 strain of influenza virus results in: 1. The decrease in the phospholipid phosphorus concentration of the membrane; 2. The presence of large amounts of lipid-bound phosphorus in the fluid medium; and 3. A more rapid uptake of P^{32} into the membrane.

The author wishes to express his sincere gratitude to Dr. Boris Magasanik for his assistance in the course of this work, and to Dr. A. K. Solomon for his help in the procurement and analysis of the radioactive materials.

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Lack of Interference of Chloramphenicol with Penicillin in a Hemolytic Streptococcal Infection in Mice. (19449)

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(Introduced by R. H. Williams.)

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Considerable interest has been shown in the recent demonstration by Jawetz and coworkers that aureomycin, terramycin, and chloramphenicol interfere with the early bactericidal action of penicillin(1-6). When any of the 3 antibiotics was combined with penicillin *in vitro*, susceptible organisms were destroyed more slowly than when exposed to penicillin alone. Interference also occurred in mice infected with group A streptococci; animals that received both chloramphenicol and penicillin had a higher mortality rate than those treated with penicillin alone. Effective blood levels were present for less than 3 hours, however, so that death or survival of the animals depended upon brief antibiotic

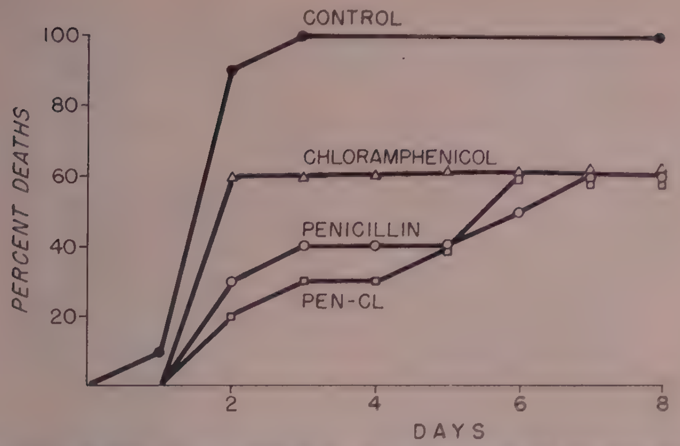

FIG. 1. Lack of interference of chloramphenicol with penicillin when treatment was continued for 3 days. Intramuscular injection of 25 units of procaine G penicillin twice daily, and 600 μg of chloramphenicol 3 times daily.

action. In other words, the animal experiments reflect the interference of chloramphenicol with the early action of penicillin.

Although the combination killed organisms more slowly *in vitro*, Jawetz also noted that the bactericidal action continued progressively until, in many instances, the cultures were sterile(4). This progressive killing action of the combinations did not play a role in the mouse experiments because of the short duration of antibiotic blood levels. However, in patients treated with antibiotics, effective levels are present in body fluids for prolonged periods. It seemed desirable, therefore, to repeat Jawetz's *in vivo* experiments under conditions that would provide more sustained antibiotic levels.

Materials and methods. Preliminary studies. Three experiments were done under the exact conditions outlined by Jawetz. Mice were infected with group A streptococci, and shortly afterward treated with a single injection of penicillin, chloramphenicol, or both antibiotics. In every case the mortality rate of the animals that received both antibiotics was significantly higher than that of those getting only penicillin. The results were clear-cut, and it was not felt necessary to pursue these confirmatory studies further. *Inoculum.* A Group A streptococcus was passed through mice twice weekly to maintain virulence. An overnight culture was diluted to 10^{-4} in broth, and each animal was given 0.2 cc intraperitoneally. Plate counts revealed that this infecting dose contained approximately 5,000 organisms, and produced a consistently high mortality rate in control animals. *Antibiotics. Chloramphenicol* was dissolved in sterile 0.85% saline in concentrations of 1.0 to 6.0 mg/cc, dispensed in sterile tubes, and stored at —20°C. It was necessary to warm mixtures containing more than 2.5 mg/cc to obtain complete solution, and these solutions usually remained supersaturated when thawed. If not, they were warmed again. *Penicillin* was administered as procaine penicillin G in peanut oil gelled with 2% aluminum monostearate in concentrations ranging from 125 to 500 units/cc.*

Procedure. The antibiotics were given in a constant volume of 0.1 cc, and were administered intramuscularly in some experiments and subcutaneously in others. It was found that somewhat smaller amounts of antibiotics were needed if the subcutaneous route was used. The animals treated with both chloramphenicol and penicillin received the 2 injections within a few seconds of one another, but at different sites. Those receiving only one antibiotic were given simultaneous injec-

* We are indebted to the Bristol Laboratories for the penicillin preparations.

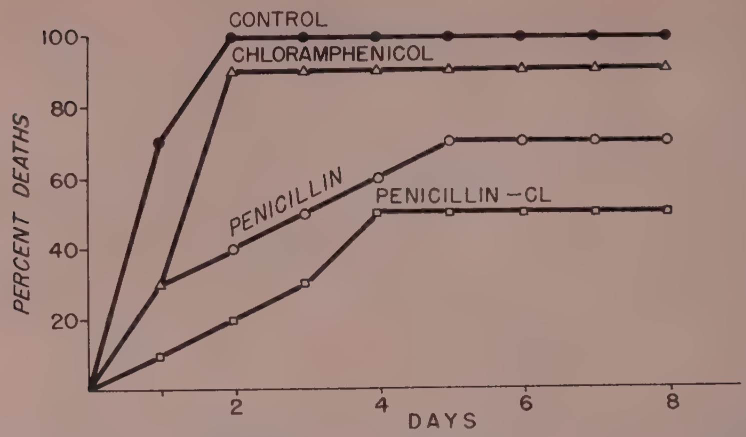

FIG. 2. No interference demonstrable with a relatively ineffective dosage of chloramphenicol (100 μg 3 times daily). Penicillin dosage as in Fig. 1. Therapy was administered intramuscularly for 3 days.

tions of sterile saline. The control animals were given no injections other than the original intraperitoneal inoculum. The chloramphenicol was injected 3 times each day at 9 a.m., 3 p.m., and 9 p.m. Penicillin was given simultaneously 3 times daily in 4 experiments, and in the other 8 penicillin was given only twice each day, at 9 a.m., and 9 p.m. Therapy was instituted one to 2½ hours after infection, and was continued for either 2 or 3 days. Deaths were recorded for 5 to 10 days following therapy. Autopsies were performed on some of the animals as they died, and pure cultures of beta hemolytic streptococci were invariably obtained from the heart's blood. Twelve experiments were performed in all.

Results. The control animals were usually dead within 72 hours. Surprisingly, an occasional animal remained entirely well. The results of a typical experiment are presented in Fig. 1. In this instance penicillin alone, and chloramphenicol alone, each protected 40% of the animals. When both antibiotics were given, the survival rate was also 40%, *i.e.*, there was neither synergism nor antagonism.

When chloramphenicol was less effective than penicillin, the mortality with the combination was approximately the same as with penicillin alone. This was observed when the amounts of antibiotics employed protected relatively few animals (Fig. 2), and also when highly effective amounts were used. In the other 10 experiments the results were essentially the same as those described. At no time was antagonism demonstrated.

Comment. Jawetz's observation of the interference of chloramphenicol with penicillin *in vivo* has been confirmed in the present studies. There was a higher mortality rate among mice treated with a single aqueous injection of both chloramphenicol and penicillin than when penicillin was administered alone. However, when significant blood levels of chloramphenicol and penicillin were maintained for 2 or 3 days, interference was not demonstrable.

The explanation for these divergent results undoubtedly lies in the *in vitro* observation that although the early killing action of penicillin is antagonized by chloramphenicol, there is a progressive bactericidal action with the combination which often results in eventual sterilization of the cultures(4). Thus, when mice were exposed to both antibiotics for only 2 or 3 hours, fewer organisms were destroyed than with penicillin alone, and the mortality rate was higher. On the other hand, when both antibiotics were present in tissues for 2 or 3 days, there was a progressive bactericidal action and, in addition, time was allowed for effective phagocytosis to occur.

Interference of any of the newer antibiotics

with penicillin appears to be of importance only when recovery is dependent upon the early, rapid killing action of penicillin. A recent report of apparent interference of aureomycin with penicillin in pneumococcal meningitis is therefore of great interest(7). This is a condition which, untreated, is associated with a high mortality rate, and in which phagocytosis is relatively ineffective(8).

The importance of the interference of the newer antibiotics with the early killing action of penicillin can be determined only by extensive clinical studies. However, on the basis of the present experiments and the evidence cited above, it is our impression that there are probably few clinical conditions in which the phenomenon is of practical importance.

Summary. 1. Mice were infected with group A streptococci and treated with chloramphenicol and penicillin, separately and in combination. Jawetz's observation that chloramphenicol interfered with penicillin when animals were given a single aqueous injection of both antibiotics was confirmed. 2. The interference was not demonstrable, however, when antibiotic blood levels were maintained for 2 or 3 days. Thus, despite early interference, the combination caused a progressive bactericidal action which, in conjunction with phagocytosis, protected the mice as well as penicillin alone. 3. These experiments imply that the phenomenon of antibiotic interference is seldom of clinical significance.

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Inhibition of Mouse Encephalomyelitis Virus, *in vitro*, by Certain Nucleoprotein Derivatives.* (19450)

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Previous work showed that various substances including heavy metals, enzyme inhibitors such as dinitrophenol, cyanide, azide and iodoacetate as well as certain amino acids and analogues inhibit the propagation of Theiler's GDVII strain of murine encephalomyelitis virus in tissue cultures of minced one-day-old mouse brain(1-3). The present report gives the results of tests with certain purines, pyrimidines and substituted pyrimidine nucleosides(4-6). Preliminary data have been presented in part elsewhere(7).

Materials and methods. Cultures were prepared in 50 ml Erlenmeyer flasks closed with rubber stoppers and containing 3 ml of Simms' X7 solution and 50-100 mg of minced mouse brain from one-day-old mice. The initial reaction of the medium was adjusted to pH 8-9. Cultures were incubated for 1-2 days at 35°-36°C. The supernatant fluid obtained after centrifugation of the pooled material from 3 flasks was tested for virus content by hemagglutination of human red blood cells with serial twofold dilutions(3).

Results. Several of the compounds tested

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TABLE I. Percentage Reduction in Hemagglutination Titers of Virus, *In Vitro*, in the Presence of Certain Compounds in Comparison with Control Cultures.

Compound tested	mg/ml	% reduction in titer
Adenine	.5	0
”	1	87–100
Adenosine	1	75
”	3	100
Adenylic acid	3	0
Cytidilic ”	3	0
Cytidine	1	0
”	3	75–87
Guanine	3	0
Guanosine	1	50
Guanylic acid	1	0
Thymine	1	0
”	3	87
Uracil	3	0
Uridine	3	0
5-Aminouridine	.2	0
”	.5	75–87
”	1	87–100
5-Bromouridine	1	50
5-Chlorouracil	1	0
5-Chlorouridine	.1	0
”	.3	50
”	.5	75
”	1	87–100
2,6-Diaminopurine	1	100
Diazouridine	.5	50
”	1	75–87
5-Formamidouridine	.5	50
”	1	50
Glucosylthymine*	1	0
Glucosyluracil*	1	0
5-Hydroxyuridine	.1	0
”	.3	75
”	.5	75
”	.7	94
3-Methyluridine	1	50
Ribosylthymine*	1	0

* Pyranose ring structure.

inhibit virus propagation (Table I). Attempts were made to reverse the inhibition of uridine substituted compounds by addition of various substances. Only uridine caused a partial reversal of inhibition (Table II). This represents the first example of what apparently is competitive metabolism found so far with this system. Guanine, 1 mg/ml, did not reverse the inhibition produced by 2,6-diaminopurine, 1 mg/ml; methionine, 3 mg/ml did not prevent inhibition by adenine, 1 mg/ml.

The effects of 5-chlorouridine, *in vivo,* on virus infection were tested in the following manner: 3 weeks old mice weighing less than 15 g each were inoculated intracerebrally with 0.03 ml of a 10^{-5} dilution of tissue culture virus (2 M.L.D.). Thirty mg of 5-chlorouridine contained in 0.5 ml volume were injected intraperitoneally 6, 30 and 54 hours after virus inoculation. The control group of mice received saline instead of 5-chlorouridine. Mice died of encephalitis on the days after virus injection as follows: test group, 2,6,6,6,6, 7,7,8,8,9; control group, 3,6,6,6,6,6,7,7,7 (1 survivor). The same results were obtained if the first dose of drug was given one-half hour before the virus injection and the second and third doses were given 24 and 48 hours later, respectively. 5-Chlorouridine had no curative effect. Larger doses were not used since it was found in other tests that a single injection of 45 mg was lethal for mice of this age.

Discussion. Some compounds which are known to interfere with nucleic acid biosynthesis also inhibit growth of bacteria and fungi(5-13). The present results show that substituted pyrimidine nucleosides, which inhibit growth of *Neurospora*(5,6) also limit propagation of Theiler's virus, *in vitro*. The strain of *Neurospora* used requires uracil, cytidine or uridine for growth. That the viral inhibition may be due to a block in the pathway of virus nucleic acid synthesis is suggested by the reversal effect produced by uridine. This was also suggested to explain the inhibition of $P^{32}O_4$ uptake by the ribonucleic acid fraction of one-day-old mouse brain in the presence of 5-chlorouridine(14). Reversal of the inhibition produced by antimetabolites on the propagation of viruses has been observed by other workers. Coliphage inhibition by 2-amino-9-(p-aminophenyl) acridinium chloride is reversed by ribonucleic acid(15). Benzimidazole or 2,6-diaminopurine inhibits vaccinia virus in chick embryo tissue culture; the latter but not the former effect is reversed by adenine(16).

An inhibitory effect on the GDVII virus propagation is observed with adenine, adenosine, cytidine, guanosine and thymine. With the exception of thymine, these compounds are utilized by the rat for tissue nucleic acid synthesis. An explanation of these results is not apparent.

Summary. Adenine, adenosine, cytidine, guanosine and thymine inhibit the propaga-

TABLE II. Partial Reversal of Hemagglutination Inhibition by Uridine Derivatives Tested with GDVII Virus in Tissue Cultures.

	% reduction of titer compared with control					
	5-Chlorouridine, 1 mg/ml		5-Aminouridine, .5 mg/ml		5-Hydroxyuridine, .3 mg/ml	
Compounds tested	Alone	Combined	Alone	Combined	Alone	Combined
Uridine, 1 mg/ml	100	75	87	60	87	50
3	87	75	—	—	—	—
Uracil, 1	94	87	87	75–87	—	—
3	—	—	75	75	—	—
Cytidine, 1	87	87	87	75	—	—
			1 mg/ml			
Uridine, 3			87	75		

tion of Theiler's GDVII strain of murine encephalomyelitis virus in tissue cultures of one-day mouse brain. Guanine, uridine, uracil, guanylic or cytidylic acids did not inhibit. Various substituted nucleosides including amino-, chloro-, diazo-, formamido-, hydroxy- and methyluridine were inhibitory. Uridine partially reversed this inhibition. 5-Chlorouracil, ribosylthymine and glucosylthymine did not inhibit; 2,6-diaminopurine did. 5-Chlorouridine was not effective against this viral infection in mice.

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Poliomyelitis Infection in Cortisone-Treated Hamsters Induced by the Intraperitoneal Route.* (19451)

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It was previously reported that cortisone enhances greatly the experimental poliomyelitis infection in the Syrian hamster(1,2). The studies embodied in this paper deal with the production of the disease in this animal by the intraperitoneal route with the aid of cortisone(3).

* Aided by a grant from the National Foundation for Infantile Paralysis.

Experimental. Syrian male hamsters weighing 20-28 g, supplied by the Lakeview Hamster Colony and the Breeding and Laboratory Institute, and Swiss mice, CF1 obtained from the Carworth Farms, weighing 15-20 g, were employed. All the animals were observed

TABLE I. MEF_1 Infection in Hamsters by the Intraperitoneal Route.

Total No.	Treatment with cortisone	MEF_1 Time of inoculation	MEF_1 Inoculum dilution	Paralysis onset Range of days	Mean survival time	Mortality rate, %
121	—	—	1:20 to 1:12000		—	0
45	2 mg 4x 24, 27, 20 hr	—	—	—	14	6
10	2 mg 1x	2 hr after cortisone	1:20	5–6	7.3	30
10	5 mg 1x	Immed. after cortisone	1:20	6–16	12	60
10	2 mg 2x 24 hr	2 hr after 2nd cortisone	1:20	5–8	7.4	100
10	2 mg 3x 19, 27 hr	Immed. after 3rd cortisone	1:1000	4–10	8	66.7
10	2 mg 4x 24, 27, 20 hr	”	1:100	3–5	4.7	100
15		”	1:500	5–10	8	100
10		”	1:1000	5–9	7.6	55.5
10		”	1:8000	5–12	9	60
10		”	1:12000	4–11	—	0
10	3 mg 3x 19, 24 hr	2½ hr after 3rd cortisone	1:20	4–7	6.5	100
10	3 mg 4x 4, 20, 3 hr	Immed. after 3rd cortisone	1:100	3–5	5.5	100

daily for a period of 4 weeks. The intracerebral inoculation dose was 0.03 ml for mice and 0.05 ml for hamsters, and the intraperitoneal dose for hamsters was 0.5 ml. All the studies described were done with strain MEF_1 supplied by Dr. Peter K. Olitsky and maintained in this laboratory by serial intracerebral mouse passages. An emulsion of a large pool of brains was preserved in an electric refrigerator at —30°C. The intracerebral mouse LD_{50} titer was $10^{-3.78}$. Since 0.5 ml was used for the intraperitoneal inoculation and the mouse titrations were based on intracerebral inoculation of 0.03 ml the doses used for the intraperitoneal inoculation are best expressed in terms of dilutions rather than in equivalent mouse LD_{50} titers until the ratios of the intracerebral mouse and intraperitoneal hamster LD_{50} titers are determined experimentally. The anti-Lansing monkey convalescent serum obtained from Dr. Hilary Koprowski and another similar serum prepared in this laboratory were used for the neutralization experiments described. The author is thankful for these investigators for making the materials available. Cortisone, Cortone acetate, Merck and Co., containing 25 mg per ml was injected intramuscularly in a volume not exceeding 0.2 ml. When smaller concentrations of the hormone were needed the dilutions were made in saline. Lyophilized ACTH, ACTHAR, Armour Laboratories, was dissolved to a suitable concentration in saline and injected in a volume of 0.25 ml intramuscularly.

Results. As may be seen from Table I, it was possible to infect hamsters consistently by the intraperitoneal route following cortisone treatment. The animals developed a rapidly progressing, highly fatal disease, the severity of which equaled and possibly exceeded that obtained on intracerebral inoculation of the virus into cortisone-treated hamsters. The results were unequivocal inasmuch as no clinical disease was elicited following the inoculation of the virus into hamsters receiving no cortisone. There was a clear-cut reciprocal relationship between the amount of cortisone and the concentration of the virus necessary for the production of the disease. The mean survival time and the mortality rate were readily influenced by these relationships, resulting under optimum conditions in an incubation period as short as 3-5 days and 100% mor-

TABLE II. Recovery of Virus from Hamsters Infected Intraperitoneally.

Treatment with cortisone	MEF_1 inoculum dilution	Organ tested — Name	Hr after inoculation	Dilution	Result in mice†
—	1:20	Brain	96	1:20	0/8
5 mg 1x	”		20	”	0/7
	”		70	”	0/8
	”		192	”	5/8
—	”	Serum	96	1:2	0/8
5 mg 1x	”		70	1:10	0/6
	”		192	”	0/6
2 mg 2x 24 hr	”		23	”	1/16
	”		23	1:100	0/16
	”		96	1:10	13/16
	”		96	1:100	2/16
3 mg 3x 19, 24 hr	”	Brain	96	” *	6/6
	”	Serum	96	1:10	11/16
	”		96	1:100	4/16
3 mg 4x 4, 20, 3 hr	1:100	Brain	120	”	6/6
	”		120	1:1000	5/6
	”		120	1:5000*	5/6
	”	Serum	120	1:10	6/6
	”		120	1:100	2/6
	”		120	1:1000	1/6
5 mg 4x 19, 24, 24 hr	1:1000		72	1:10	6/6
	”		72	1:100	3/6
	”		72	1:1000	0/6
	”		120	1:10	6/6
	”		120	1:100	6/6
	”		120	1:1000	5/6
	”		120	1:4000	1/6

* Higher dilutions not tested.
† Numerator = No. of dead animals. Denominator = Total No. of animals tested.

tality rate. It is of interest that a single injection of 5 mg was less effective than 2 injections of 2 mg given at an interval of 24 hours. Four injections of 2 mg gave rise to a fatal disease following the inoculation of 0.5 ml of the virus diluted as high as 1:8000. Even after taking into consideration the fact that a volume 10 times greater was employed for the intraperitoneal than for the intracerebral inoculation, it may still be assumed that the intraperitoneal route compares quite favorably with the intracerebral route in cortisone-treated hamsters.

The identity of the disease was established as follows: 1. There was obtained a typical clinical disease in a great majority of animals frequently resulting in tetraplegia in animals surviving for at least 4 days. Characteristic histological changes were invariably seen in the spinal cord in the animals showing the disease during life. 2. The virus recovered from the brain of the intraperitoneally infected hamsters produced typical symptoms in mice. It was neutralized by the anti-Lansing convalescent monkey serum on intracerebral testing in mice. In these titrations 200 mouse LD_{50} were completely neutralized by the serum diluted 1:10 following incubation of the mixture for one hour in a water bath at 37°C. 3. It was also possible to neutralize the virus in the hamsters. In these experiments the hamsters received 4 injections of 2 mg of cortisone at intervals of 3, 20, and 3 hours. One-half ml of suitable dilutions of virus and serum incubated in a water bath at 37°C for one hour were injected intraperitoneally immediately following the fourth injection of cortisone. There was obtained consistent neutralization of the virus diluted 1:500 with the serum diluted 1:40, while in

the absence of the serum the virus similarly diluted and incubated gave 100% mortality with a mean survival time of 5 days.

As may be seen from Table II, the virus multiplied in the central nervous system incidentally to the development of the disease. In addition the animals showed a marked viremia, the extent of which depended largely on the total dose of cortisone, the frequency of its administration and the concentration of the virus. The proof that the viremia obtained was not due to the discharge of the virus into the vascular system from the central nervous system through breaking down of some barrier by means of cortisone was found in the observation that hamsters similarly treated with cortisone and inoculated with the virus intracerebrally failed to show any evidence of viremia at different stages of the disease.

It was previously noted that in contrast to cortisone, ACTH in large doses failed to modify the poliomyelitis infection in the hamster inoculated with the virus intracerebrally(1,2). Similarly 15-20 mg of ACTH injected intramuscularly in 3 and 4 divided doses over a period of several days failed to elicit the infection by the intraperitoneal route in 40 hamsters inoculated with 0.5 ml of strain MEF_1 diluted 1:20. All the animals thus tested remained well during the entire period of observation.

Discussion. The results reported in this paper indicate an extraneural multiplication of the poliomyelitis virus following the intraperitoneal inoculation into cortisone-treated hamsters, since the viremia observed fails to appear following the intracerebral inoculation of the virus into hamsters treated similarly with cortisone. Unpublished work in collaboration with Dr. S. M. Aronson indicates a good correlation exists between histological changes and concentration of the virus in certain organs and tissues outside of the central nervous system. Among these are changes in periadrenal adipose tissue and necrotizing calcified lesions in paravertebral muscles incidentally to a high concentration of the virus. The results are of special interest in connection with the pathogenesis of the disease because they suggest that extraneural multiplication of the virus may be one of the phases of the infection on parenteral introduction of the virus. The demonstration by Horstmann(4) and Bodian(5) of a viremia in the chimpanzee following feeding of the virus, points in the same direction.

The possibility of producing the infection by the intraperitoneal route offers a new method for intraperitoneal testing of neutralizing potency of sera and for investigations of the effect of chemotherapeutic agents before fixation of the virus by the central nervous system. Other distinct advantages of this method result from the fact that by adjusting the dosage of cortisone and the concentration of the virus it may become possible to obtain various degrees of severity of the disease ranging from a prolonged incubation period and low mortality rate to a uniformly severe disease of short incubation period and high mortality rate, thus affording the opportunity for screening of agents under widely different conditions.

Summary. It was possible to produce a violent and uniformly fatal poliomyelitis infection in the hamster by the intraperitoneal route following treatment with cortisone. The disease was accompanied by a pronounced viremia apparently due to the multiplication of the virus outside of the central nervous system under these conditions. No clinical infection was observed in non-treated hamsters thus inoculated. No disease was produced following the intraperitoneal inoculation of the virus in the hamsters receiving ACTH.

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Photometric Assay of Plasma Heparin.* (19452)

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Chemical methods for the quantitative determination of heparin in blood plasma are based on the changes in certain basic dyes added to at least partially separated metachromatic substance. Heparin seemingly is not found in normal blood plasma, though traces of other mucoitin sulfates are present. It is possible to follow by heparin estimations the plasma level when heparin is administered and to recover heparin added to blood plasma. Gibson *et al.*(1) reported briefly a separation and photometric procedure for estimating heparin in blood plasma. Further details and some applications are now presented. The concentration of heparin is expressed as anticlotting units in terms of the weight of certain purified standards, *e.g.*, those of the International Provisional Standard and of the Connaught Laboratory. The U. S. Pharmacopoeia now defines a unit as approximately the quantity of heparin sodium required to maintain fluidity in 1 ml of plasma prepared according to the directions of the U.S.P. assay for heparin sodium. We are using for a standard an Upjohn heparin sodium assaying 130 units per mg. Metachromatic heparin assay was proposed by MacIntosh(2) who used purified preparations in concentrations of about 0.01% and a provisional British standard. He measured the loss of color of an aqueous toluidine blue O solution (with a Spekker absorptionometer) after separating the red precipitate by overlaying the mixture with petroleum ether. Jaques, Mitford and Ricker(3) assayed solutions of heparin prepared according to Charles and Scott(4), measuring the change in color of azure-A with a Lovibond tintometer. Jaques, Morehouse and Stewart(5) precipitated the "heparin-protein-complex" from citrated plasma with n-octylamine, reprecipitated the heparin with brucine phosphate, and quantitated it as above. Good recoveries of added heparin were obtained, but with small quantities and with normal plasma difficulties were encountered. Then Monkhouse and Jaques(6) adapted a phenol separation of heparin from plasma devised by Homan and Lens(7) for metachromatic assay. The metachromatic assay of purified heparin offers little difficulty. The addition of toluidine blue O or azure A to a heparin sodium solution (pH 7.4-7.8) results in a flocculation of a reddish purple precipitate. This can be centrifuged and the precipitate washed and extracted with alkaline alcohol. The resulting red solution may be read photometrically.

Method. A separation of the heparin from plasma or serum is essential. The following procedure when carefully followed is satisfactory. *Special reagents.* n-octylamine (Sharples) 2 ml, concentrated hydrochloric acid 1 ml, and water 30 ml, adjusted to a pH of 7.3-7.5; 0.5 N sodium hydroxide and exactly 8.05% (0.5N) zinc sulfate; toluidine blue O solution, 100 mg in 100 ml of water, diluted 4:10 and centrifuged before using.

Five ml of oxalated plasma in a 15 ml test tube are diluted with 5 ml of pH 6.1 M/15 phosphate buffer. Then 2 ml of n-octylamine hydrochloride solution are added in 0.5 ml

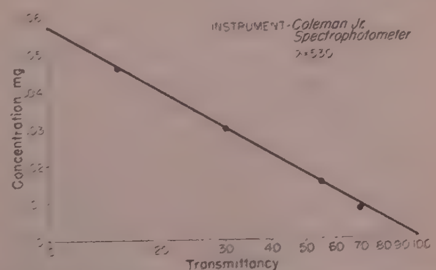

FIG. 1. Standard curve, Upjohn, sodium heparin (130 units per mg).

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portions at 5-minute intervals with immediate mixing. The tube is centrifuged as soon as flocking is observed. If flocking is delayed over 10 minutes, the tube may be warmed to 40°C. The tube is centrifuged, the supernatant poured off, and the precipitate washed by suspension in 10 ml of water and again centrifuged. The precipitate is moistened with exactly 0.5 ml of 0.5 N sodium hydroxide solution, mixed with 10 ml of 0.9% saline and heated at 60-70°C in a water bath for 15 minutes. When cool, 0.5 ml of 0.5 N zinc sulfate solution is added and mixed and the tube again centrifuged after 30 minutes. The heparin is contained in the precipitate. It is extracted with 5 ml of water plus 5 ml of 7.8 phosphate buffer and heated at 65-70°C with stirring for 15 minutes. The tube is centrifuged and the supernátant drained into a fresh test tube. To this is added 1 ml of the toluidine blue O solution. A reference blank with water-buffer mixture and dye is made. Both tubes are allowed to stand for several hours, or better overnight. A red precipitate forms in the heparin tube. The heparin tube is centrifuged, drained, and the red precipitate washed in 5 ml of water and again centrifuged. The reference tube is similarly washed. The inside walls of both tubes after draining are swabbed with alcohol-moistened and dry gauze sponges. Four ml of alcohol and 1 ml of 0.1 N sodium hydroxide are added to each tube. After 20-30 minutes the heparin tube is centrifuged and the supernatant read at 530 mμ in a Coleman Jr. spectrophotometer (75 x 12 mm cuvettes) with the dye blank tube set at 100% transmittancy.

A calibration curve for heparin sodium is prepared. The standard should contain 10 ml of heparin sodium (Upjohn) in 100 ml of saline 0.9%. Four portions: 0.01, 0.02, 0.03 and 0.04 mg of heparin sodium are added to 10 ml amounts of saline and precipitated with sodium hydroxide and zinc sulfate. The heparin is extracted and precipitated and the colored end solution obtained as above. The graph is a straight line (Fig. 1) when plotted on semi-log coordinates.

The metachromatic precipitate. Heparin enters into a combination with the dye to form the red-purple precipitate. If the red alkaline alcohol solution for the calibration curve is centrifuged, the trace of residue when taken up in phosphate buffer can be reprecipitated with the dye and again gives the red color of the same intensity with alkaline alcohol. This procedure may be repeated and further purification of heparin seems possible.

TABLE I. Recovery of Heparin Sodium Added to 5 ml of Plasma.

Plasma, mg	+.01 mg, mg	Recovered, mg
.011	.022	.011
.015	.024	.009
.003	.013	.010
.006	.019	.013
.027	.036	.009
.013	.025	.012
.008	.019	.011
.012*		.011*
	+.02 mg	
.006	.026	.020
.011	.029	.018
.006	.024	.018
.006	.025	.019
.002	.021	.019
.005	.026	.021
.005	.024	.019
.006*		.019*
	+.03 mg	
.008	.032	.024
.005	.036	.031
.003	.035	.032
.016	.045	.029
.010	.040	.030
.007	.037	.030
.007	.033	.026
.008*		.029*
	+.04 mg	
.013	.052	.039
.007	.050	.043
.005	.047	.042
.001	.039	.038
.010	.048	.038
.012	.055	.043
.012	.055	.043
.006*		.041*

* Average.

Recovery of heparin added to plasma. Determinations on 5 ml amounts of normal plasma to which had been added .010, .020, .030 and .040 mg of heparin sodium were done. Recoveries have been very satisfactory, averaging .011, .019, .029, and .041 mg for the 4 groups (Table I).

The normal plasma level of metachromatic substance as heparin sodium. Metachromatic

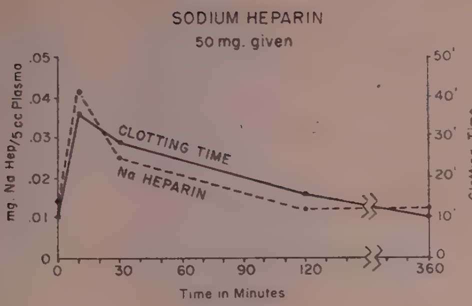

FIG. 2.

changes are induced by other mucoitin sulfates and esters (Lison)(8). Monkhouse and Jaques(9) are of the opinion that the metachromatic material in normal blood is not heparin. Significant amounts of metachromatic reacting substances are found in normal plasma, as may be seen from the plasma figures in Table I. These have amounted to .02 to .54 mg%. Phosphate saline extracts of 20 ml of pooled plasma were concentrated *in vacuo* and dialyzed against saline solution to remove the phosphates, and the anticoagulant activity tested by the Waugh-Ruddick(10) technic. There was no increase in the clotting time. If heparin was added to the plasma similarly treated, the heparin was recovered when assayed by effects on clotting time.

Plasma heparin levels and clotting times. The curve in Fig. 2 shows the plasma heparin sodium levels for 5 ml plasma samples and clotting times when heparin sodium was administered i.v., in a 50 mg dosage. The curve for the heparin contents parallels the clotting times. Depo-heparin 200 mg intramuscularly exhibits a prolonged but lesser clotting time increase and only a slight rise of the heparin level.

Urine. It has long been known that urine contains some chondroitin sulfuric acid in its non-dialysable constituents. Pons(11) has determined the daily output in the urine as 80 to 90 mg per day. If 1 ml of normal urine is diluted to 10 ml with water, precipitated with sodium hydroxide and zinc sulfate, the precipitate treated with pH 7.8 phosphate and water and toluidine blue O added to the supernatant, a considerable metachromasia is obtained. This amounts to 3 to 12 mg% expressed as heparin sodium. When equal parts of 3.5% saline instead of water and the pH 7.8 phosphate are used, there is no metachromatic effect with urine alone, but heparin added to the urine can be determined. For example, 1 ml of the urine treated as above gave by the water-buffer-dye precipitation .060 mg of metachromatic substance expressed as heparin sodium; with .025 mg of added heparin sodium, .084 mg was found. The 3.5% saline and buffer procedure was .00 mg for the urine, but .025 mg of added heparin sodium returned .026. A control with .025 mg heparin sodium assayed .026 mg. Astrup(12) used a benzidine precipitation of the heparin-like substance in urine (and plasma). This substance has a slight anticlotting effect, which he did not believe to be heparin. We have found little or no anticlotting effect in our metachromatic reacting material from normal urine.

Summary. A photometric assay of heparin sodium is described. This consists of a preliminary precipitation of 5 ml of plasma with n-octylamine, reprecipitation with zinc hydroxide, decomposition of this precipitate with phosphate buffer solution, and final precipitation with toluidine blue O. After washing, the red-purple residue is taken up in alkaline alcohol and read photometrically. The heparin is a component of the red-purple dye precipitate. Normal plasma contains non-active heparin-like substances, amounting to 0.02 to 0.54 mg%. Added heparin sodium to plasma is quantitatively recovered. Intravenous heparin sodium and "depo-heparin" levels follow the clotting inhibition effects. Normal urines contain from 3 to 12 mg% of non-active metachromatic substance expressed as heparin sodium. Heparin sodium added to urine may be recovered.

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Effects of Vitamin A Malnutriture on Resistance to Stress.* (19453)

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Considerable data are available indicating that resistance to stress may be significantly impaired in the nutritionally-deficient animal. Thus an impaired resistance to cold has been demonstrated in rats deficient in pyridoxine (1), riboflavin(2), and vit. A(3,4), and guinea pigs deficient in vit. C(5). An impaired resistance to anoxia has been reported in the riboflavin-deficient rat(6,7); and failure to excrete water and to withstand water intoxication has been observed both in riboflavin-deficient(8) and pyridoxine-deficient (9) rats. In the present communication data are presented on the effects of graded doses of vit. A on resistance to 1) low environmental temperature and 2) x-irradiation injury in the rat.

Procedure. The animals employed in the present experiment were male rats of the University of Southern California strain. The mother rats were maintained for at least 2 months previous to breeding and for 12 days after the birth of the litter on Sherman diet B(10) without addition of supplementary lettuce or meat. Litters were cut to 7 at 3 days of age, and on the 12th day mothers and litters were placed on a vit. A-low diet.† The young were weaned at 21-24 days of age and at a body weight of 36-50 g inclusive. The basal ration employed in the present experiment had the following composition: sucrose, 60%; casein,‡ 25%; salt mixture,§ 5%; cottonseed oil (Wesson), 10%; and the following synthetic vitamins per kg of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; ascorbic acid, 200 mg; biotin, 5 mg; folic acid, 10 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12}, 150 μg; 2-methyl-naphthoquinone, 10 mg; and choline chloride, 2 g. To each kg of diet were also added 400 U.S.P. units of vit. D.‖ Each rat also received once weekly a supplement of 4.5 mg alpha-tocopherol acetate. Animals were kept in metal cages with raised screen bottoms to minimize access to feces and were fed *ad libitum*. Diets were made up weekly and stored under refrigeration. Rats were fed on alternate days. All food not consumed 48 hours after feeding was discarded to minimize oxidative changes in the diet.

Results. Exp. 1. Effects of graded doses of vit. A on survival time of rats under cold room and room temperature conditions. Rats averaging 43.4 g in weight were divided into 5 groups of 30 each and were fed the basal vit. A-free ration. They received in addition,

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† Similar to U.S.P. XIII depletion diet except that commercial casein rather than extracted (vit. A-free) casein was employed.

‡ Vitamin Test Casein, General Biochemicals, Chagrin Falls, O.

§ Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

‖ HY-DEE Powder, Standard Brands, New York.

TABLE I. Effects of Graded Doses of Vit. A on Growth and Survival of Rats Under Cold and Room Temperature Conditions.

Avg daily supplement of vit. A, U.S.P. units	No. of animals	Initial body wt, g	Gain in body wt, 30 day period, g*	% surviving	Avg survival of decedents, days†
Cold room series, $2 \pm 1.5°C$					
0	15	111.5	—	0	$1.9 \pm .6$
2.5	12	153.5	31	58	16.2 ± 2.7
5	12	156.4	20.8	92	$10 \pm .0$
10	12	154.3	24.9	100	—
50	12	163.7	34.1	100	—
Room temperature series, 23°C					
0	15	105.1	—	0	19.3 ± 1.2
2.5	18	151.7	82.7	100	—
5	18	152.6	93.7	100	
10	18	149.6	105.1	100	—
50	18	163.4	109.9	100	—

* Of surviving animals.

† Including stand. error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

twice weekly by graduated syringe, oral supplements of vit. A¶ dissolved in 0.12 cc of cottonseed oil in amounts furnishing an average daily intake of 0, 2.5, 5, 10, and 50 U.S.P. units of vit. A for groups I to V, respectively. Rats in group I became depleted of vit. A (as judged by stationary or decreasing body weight for a period of 5 days) after an average of 23.4 days of feeding (range 21 to 27 days) and at an average body weight of 108.3 g. At the time of depletion half the animals were placed (1 animal per cage) in a walk-in refrigerator maintained at a temperature of $2° \pm 1.5°C$. The remaining half were continued at standard laboratory conditions (23°C). After 24 days of feeding the average body weight of rats in the remaining dietary groups was as follows: group II, 152.3 g; group III, 154.0 g; group IV, 151.3 g; and group V, 163.5 g. At this time animals in the above groups were divided into 2 series. Eighteen rats in each group were continued at standard laboratory conditions; the remaining 12 animals were placed in the walk-in refrigerator (1 animal per cage). No change was made in the diets fed. Feeding was continued *ad libitum* for an additional 30 days or until death, whichever occurred sooner. Results are summarized in Table I.

In agreement with earlier findings(3,4), exposure to a low environmental temperature significantly decreased the survival time of rats depleted of vit. A. At an environmental temperature of $2 \pm 1.5°C$, the average length of survival of rats depleted of vit. A was 1.9 days in contrast to an average survival time of 19.3 days for depleted rats at room temperature. An average daily intake of 2.5 U.S.P. units of vit. A was insufficient to provide maximal adjustment to cold since 42% of the rats in this series succumbed during an experimental period of 30 days. An average daily intake of 5 U.S.P. units of vit. A was sufficient, however, to permit an apparently optimal adjustment to cold. Under room temperature conditions all rats fed an average daily intake of 2.5 U.S.P. units of vit. A or more survived. Gain in body weight was correlated at room temperature conditions with the amount of vit. A administered, being significantly greater for rats fed the higher doses of vit. A than the lower. The differences between the various groups, however, were not marked; and with the exception of size the various groups were grossly indistinguishable. It is apparent from these findings that an average daily intake of 2.5 U.S.P. units of

¶ Vit. A Palmitate (Synthetic), Hoffmann-La Roche, Nutley, N. J.

vit. A, although sufficient for good growth and survival under room temperature conditions, was inadequate for optimal adjustment to cold.

Exp. 2. Effects of graded doses of vit. A on mean arterial pressure of rats maintained under cold and room temperature conditions. At the termination of Exp. 1, 6 rats were selected at random from each of the surviving groups and the mean arterial pressure determined by the direct method.** The rats were anesthetized with ether and the abdominal aorta was exposed and punctured with a 24 gauge needle connected to a mercury manometer of 1 mm bore tubing. The puncture was made during light anesthesia and with the mercury column in the manometer raised to a level of 90-100 mm Hg. In the cold room series the average mean arterial pressure was 108.3, 95.4, 95.8, and 91.4 mm Hg, respectively, for rats fed an average daily intake of 2.5, 5, 10, and 50 U.S.P. units of vit. A. In the room temperature series the average mean arterial pressure of these groups was 101.5, 91.8, 95.4, and 98.2 mm Hg, respectively. The differences observed between rats fed the varying levels of vit. A were not statistically significant either under cold room or room temperature conditions; nor were the differences between the 2 environmental series on any of the rations employed. The latter findings are at variance with the report(11) that prolonged exposure to cold causes a significant increase in the systolic blood pressure of the rat. Gilson's determinations, however, were made with a tail plethysmograph on unanesthetized rats fed a natural food ration. In unpublished findings from this laboratory it was observed, in agreement with Gilson, that prolonged exposure to a low environmental temperature resulted in a significant increase in the systolic blood pressure of rats on a natural food ration, as determined in unanesthetized animals with a Sobin tail plethysmograph. When the mean arterial pressure was determined on these same rats, however, by the direct method outlined above, no increase in mean arterial pressure was observed over that of animals fed a similar ration but maintained under room temperature conditions. It would appear, therefore, either 1) that the hypertension caused by prolonged exposure to cold is abolished by ether anesthesia or 2) that the tail plethysmograph is not an accurate method for determining the systolic blood pressure of rats which had been exposed to a low environmental temperature for a prolonged period of time.

Exp. 3. Effects of graded doses of vit. A on survival of rats administered a single lethal dose of x-irradiation. At the termination of Exp. 1, 10 rats were selected at random from each of the surviving groups in the room temperature series and were administered a single dose of 750 r x-irradiation. Animals were continued on the basal ration plus their respective vit. A supplements for 30 days post-irradiation. The radiation factors were as follows: GE Model Maximar 250; 250 KV; 15 MA; 0.5 mm Cu and 1 mm Al filters plus a Cu parabolic filter;†† HVL, 2.15 mm Cu; target distance to top of box, 78 cm; and dose rate, 17.92 r per minute (measured in air). The animals to be irradiated were placed in a wooden box divided into 14 compartments 7 cm wide, 16 cm long and 10 cm deep (10 rats were irradiated at a time). The partitions and top were made of ⅛-inch cellulose acetate sheeting; and the top, one side and bottom of each compartment were perforated with holes for purposes of ventilation. The container was rotated slowly on an electrically-driven turntable to insure equivalent irradiation. The per cent survival and the average survival time of decedents in the various groups are summarized in Table II.

A significant difference was observed in the survival rate of rats fed varying doses of vit. A following a single exposure of 750 r

** We are indebted to Dr. Sheldon Rosenfeld, Dept. of Physiology, University of Southern California, for technical assistance in the blood pressure determinations.

†† A non-uniform filter which produces a flat isodose surface of x-ray intensity constructed by the method of Greenfield and Hand(12). We are indebted to Dr. Moses Greenfield and Miss Katherine Hand of the Atomic Energy Project, University of California at Los Angeles, for construction of the parabolic filter. The center of the filter had a thickness of 1.7 mm Cu; the edge, 0.5 mm Cu.

TABLE II. Effects of Graded Doses of Vit. A on Survival of Rats Administered a Single Lethal Dose of X Irradiation. 10 rats in each series.

Avg daily supplement of vit. A, U.S.P. units	Initial body wt, g	Change in body wt, 30 days post-irradiation, g*	% surviving	Avg survival of decedents, days†
2.5	217.4	—	0	8.8 ± .8
5	276.3	−17.8	50	18.5 ± 3.8
10	279.3	−.4	50	14.4 ± 2.3
50	292.7	10.9	90	9 ± .0

* Of surviving animals. † Including stand. error of the mean

x-irradiation. All of the rats receiving 2.5 U.S.P. units of vit. A daily succumbed, whereas, 50% of the rats fed 5 or 10 U.S.P. units, and 90% of those on 50 U.S.P. units of vit. A survived. During the first 4 days post-irradiation, rats in all groups lost approximately 20-25 g in body weight. Surviving animals fed the higher doses of vit. A gained back the weight they had initially lost. This did not occur, however, in rats fed an average daily intake of 5 U.S.P. units of vit. A. An unexpected finding was the occurrence of circular necrotic patches on the tail of irradiated rats fed an average daily intake of 5 or 10 U.S.P. units of vit. A (Fig. 1). These first became manifest during the second week post-irradiation and persisted for approximately 2 weeks in those animals which survived. Seventy percent of the rats fed an average daily intake of 5 U.S.P. units of vit. A and 90% of those receiving an average daily intake of 10 U.S.P. units of vit. A developed such patches. No such patches were observed in any of the rats receiving an average intake of 50 U.S.P. units of vit. A daily. Rats receiving an average daily intake of 2.5 U.S.P. units of vit. A succumbed following x-irradiation without showing such lesions.

Discussion. Results of the present experiment indicate that doses of vit. A sufficient to promote good but suboptimal growth under standard laboratory conditions are inadequate for optimal resistance to stress as caused by 1) prolonged exposure to a low environmental temperature or 2) a single exposure of 750 r x-irradiation. No data are available to indicate what factors were responsible for the impaired resistance of vit. A-deficient rats to the above stressor agents. Since food intake was reduced in the deficient rats, it is possible that the decreased resistance of these animals was due, at least in part, not to vit. A malnutriture *per se* but the attendant reduction in caloric intake. Further experiments are

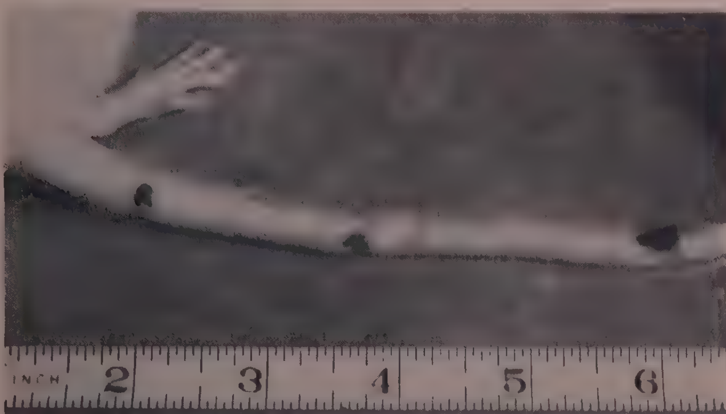

FIG. 1. Rat tail, 18 days post-irradiation, of animal fed a purified basal ration supplemented with average daily dose of 10 U.S.P. units of vit. A.

indicated to determine to what extent this factor may have contributed to the observed results.

Summary. Experiments were conducted on the effects of graded doses of vit. A on resistance to low environmental temperature and x-irradiation injury in the male rat. The average survival time of rats depleted of vit. A was 1.9 days at an environmental temperature of 2°C and 19.3 days under standard laboratory conditions (23°C). Adjustment to low environmental temperature (as judged by per cent surviving an experimental period of 30 days) was significantly increased in animals fed an average daily intake of 2.5 U.S.P. units of vit. A, but a minimal intake of approximately 5 U.S.P. units of vit. A daily was required for optimal adjustment to cold. A direct correlation was observed between the vit. A content of the diet and resistance to x-irradiation. All rats administered an average intake of 2.5 U.S.P. units of vit. A daily succumbed following a single exposure of 750 r x-irradiation; 90% of the rats receiving a daily intake of 50 U.S.P. units of vit. A and exposed to a similar dose of x-irradiation survived. An average daily intake of 10 U.S.P. units of vit. A was inadequate for optimal resistance to x-irradiation, although this level of vit. A was adequate under standard laboratory conditions for optimal growth.

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Reduction of Urinary Sodium and Potassium Produced by Hypophyseal Growth Hormone in Normal Female Rats.* (19454)

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Previous work from this laboratory(1) has shown that the administration of hypophyseal growth hormone (GH) to diabetic male rats results in the retention of both sodium and potassium even though the degree of glycosuria may be increased concurrently. It is the purpose of this paper to report that GH produces both sodium and potassium retention in normal female rats.

Methods. Three experiments are reported in this paper designated as Series I, II, and III. In each experiment 10 "plateaued" female rats of the Long-Evans strain were used. Five animals of each group were fed the regular stock diet† and 5 received the same stock diet plus an additional 600 mg of KCl

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† The stock diet consisted of ground whole wheat 67.5%, casein 15.0%, whole milk powder 10.0%, NaCl 0.75%, $CaCO_3$ 1.5%, hydrogenated vegetable oil, 5.25%. To each kg of diet were added 3.5 g Sardilene (fish oil concentrate containing 3000 USP units of vit. A and 400 chick units of vit. D per g).

TABLE I. Effect of Growth Hormone (GH) on Urinary Na and K of Normal Rats.*

		Urinary sodium				Urinary potassium			
Series	Diet	Pre-injection control period	GH period	Change	p§	Pre-injection control period	GH period	Change	p§
1	Control	2.2±.01† (30)‡	1.8±.07 (28)	–.4	<.01	1.19±.002 (30)	.95±.002 (28)	–.24	<.01
1	High KCl	2.5±.01 (30)	2.1±.01 (29)	–.4	”	7.3 ±.3 (19)	6.98±.18 (33)	–.33	”
2	Control	1.6±.03 (20)	1.2±.02 (20)	–.4	”	1.24±.02 (20)	.99±.01 (20)	–.25	”
2	High KCl	1.6±.01 (25)	1.4±.02 (20)	–.2	”	6.63±.04 (25)	6.12±.01 (25)	–.51	”

* Values expressed as mEq/rat/day. † Stand. dev. of the mean: $\sigma = \sqrt{\dfrac{\Sigma d^2}{n(n-1)}}$.

‡ No. of observations in group. § Comparing the GH period with pre-injection control period. p from Fisher's(9) table of t where

$$t = \frac{M_1 - M_2}{\sqrt{\left[\dfrac{\Sigma d_1^2 + \Sigma d_2^2}{n_1 + n_2 - 2}\right] \times \left[\dfrac{1}{n_1} + \dfrac{1}{n_2}\right]}}$$

per rat per day. The animals were kept in individual metabolism cages and urines were collected daily. The food intake for each animal was constant from day to day throughout the experiment. The methods for collection of specimens, for analyses, and for maintaining the constancy of the dietary intake were the same as those previously reported (1,2). The GH was prepared by one of us according to the previously published method (3) and the same lot of hormone was used throughout. In each case the GH was given twice daily by intraperitoneal injection. The animals in Series I received 500 γ of GH per day for a 7-day injection period which followed a 6-day pre-injection control period, and was followed by a 5-day post-injection control period. The animals in Series II received 300 γ of hormone per day for a 5-day injection period following a 5-day control period. In Series III following a 7-day control period, the animals received initially 25 γ of GH per day for two days, then 50 γ per day for 2 days, then 75 γ for one day, then 150 γ per day for 2 days, and finally 1,000 γ per day for 2 days.

Results and comments. The pertinent data regarding urinary electrolytes are presented in Table I and Fig. 1. The data in Table I show that in both Series I and II there was a significant reduction of urinary Na and K during GH treatment. Significant reduction in the urinary nitrogen also occurred in all groups of animals in Series I and II, and there was no difference between the animals on the control diet and the high KCl diet in this respect. This was in contrast to two previous experiments in which there was significantly greater nitrogen retention produced by GH in the animals on the high potassium diet. Inspection of the data presented in Fig. 1 shows that the diminution of urinary Na and K produced by GH administration was present with doses of hormone as low as 75 γ per day.

That GH causes concurrent K and nitrogen retention is not surprising since both K and protein are essential intracellular components. The retention of Na is probably associated with an expansion of the extracellular fluid volume. This hypothesis is in accord with the demonstration(4) that GH administration to hypophysectomized rats is associated with an increase in the thiocyanate space but does not produce an increase in the muscle Na or any change in serum Na. That these changes in urinary sodium and potassium excretion reflect an actual positive balance for these elements is shown by the fact that fecal analy-

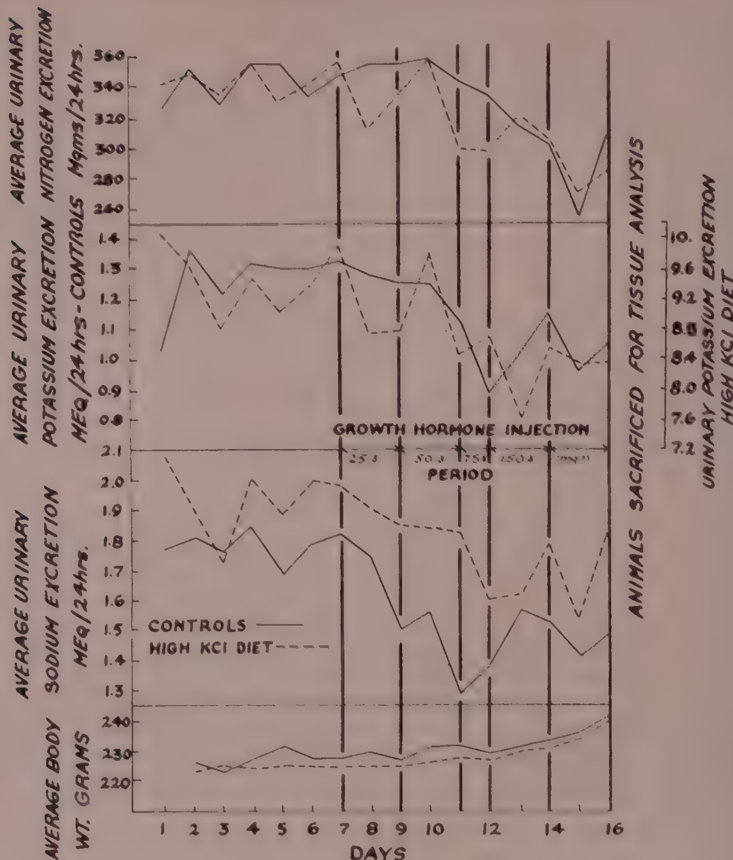

FIG. 1. Effect of growth hormone on average urinary nitrogen, potassium and sodium excretion and body wt of rats fed a high KCl diet.

ses revealed no change in fecal Na or K during the period of GH administration. This was demonstrated in an additional series of experiments which are not reported in detail here although significant reduction of urinary Na and K occurred in these experiments also. It is unlikely that unrecognized contamination of the GH could have been responsible for these results. The GH preparation used was free of antidiuretic activity as assayed by the Ham and Landis(5) procedure at a maximum dose level of 500 γ. At a dose level of 1 mg per day it was free of ACTH activity as measured by the lack of increase in the adrenal weight of hypophysectomized rats(6). A dose of 100 γ in hypophysectomized rats did not cause depletion of adrenal ascorbic acid by the procedure of Sayers *et al.*(7), again indicating that the preparation was substantially free of ACTH contamination. Even if ACTH contamination were present it is unlikely that it would have caused these electrolyte effects, since in our hands and in those of others(8) ACTH has not caused sodium retention in normal rats although it causes potassium loss, the opposite of the effect of GH on potassium balance.

Conclusion. The administration of hypophyseal GH to normal female rats produces retention of both sodium and potassium.

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Study of Ureidosuccinic Acid and Related Compounds in Pyrimidine Synthesis by *Lactobacillus bulgaricus* 09. (19455)

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Lactobacillus bulgaricus 09 has been found to respond to orotic acid as a growth factor (1-4). Several intermediates in the chemical synthesis of orotic acid have been tested and ureidosuccinic acid was shown to have from 10-20% of the activity of orotic acid(2). In a study of certain natural products it was found that human urine gave a response equivalent to about 100 γ of orotic acid per milliliter(2). Isolation studies in these laboratories have revealed that the microbiological activity of urine is due to urea.

The utilization of urea by *L. bulgaricus* raises the question as to whether or not the organism utilizes only the urea portion of the ureidosuccinic acid molecule in the synthesis of pyrimidines. Both orotic acid and ureidosuccinic acid are utilized for the biosynthesis of pyrimidines as shown by tracer studies(5). In these studies it was demonstrated only that the ureide carbon of ureidosuccinic acid is incorporated into pyrimidines of nucleic acids. Supporting evidence is presented at this time to show that in all probability the entire molecule of ureidosuccinic acid is utilized by *L. bulgaricus* 09 in pyrimidine synthesis.

Experimental. The compounds were tested for orotic acid-like activity by the method of Wright *et al.*(2), with one exception namely that the basal medium contained no added uracil. All compounds were tested alone, but in order to obtain information as to any possible antagonistic or enhancing activity, some were tested in 2 additional ways, with orotic acid at levels of 20 γ per tube and with ureidosuccinic acid at levels of 100 γ per tube. Control tubes of orotic acid were run at levels from 0 to 100 γ per tube and ureidosuccinic acid from 100 γ to 500 γ per tube. Ureidosuccinic acid also was tested using aseptic addition to detect the possibility of structural alteration during autoclaving. No difference was found between the results obtained by the two methods. The compounds tested and the results obtained are shown in Table I. The ureides were prepared by the usual method(6) of treating the appropriate amino acid with an equivalent amount of potassium cyanate in a concentrated aqueous solution, evaporating to a syrup, or in some cases, to dryness on a steam bath, redissolving in water, acidifying with concentrated HCl with cooling and recrystallizing the product from boiling water. The succinyl thiourea was prepared by the dry fusion of succinic anhydride and thiourea (7). The 3-acetyl 2-thiohydantoin was prepared from glycine and potassium thiocyanate(8).

Results. Of the compounds tested, ureidosuccinic acid was the only compound with activity comparable to orotic acid. Urea showed a greater activity than most compounds tested. No significant enhancing effects were noted, and only thiourea showed any antagonism.

Discussion. A comparison of the various ureide structures with that of orotic acid readily shows that ureidosuccinic acid most easily meets the structural requirement as a precursor of orotic acid. That urea itself is

TABLE I. Response of *L. bulgaricus* 09 to Various Ureides and Related Compounds.

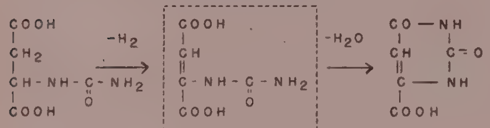
Compound	Range tested (mg/tube)	Activity (% orotic acid)	Antagonism
Orotic acid	.02—10	100	None
Urea	.2 —10	.25	
Ureidoacetic acid	.2 —10	<.1	
α Ureidopropionic acid	.2 —10	<.1	
β ” ”	.2 —10	.2	
α Ureidobutyric ”	.2 —10	<.1	
Ureidosuccinic ”	.1 — .5	10–20	
α Ureidoglutaric ”	.2 —10	<.2	
α Ureido-B methyl valeric acid	.2 —10	.0	
Fumaric acid diamide	.2 —10	<.2	
Thiourea	.2 —10	<.2	Slight at high level*
Succinyl thiourea	.2 —10	.0	”
3-Acetyl 2-thiohydantoin	.2 — 1	.0	None
Dihydro-orotic acid	— 5	.5	”

* Against ureidosuccinic acid and orotic acid at the given levels of these compounds.

relatively inactive is strong evidence alone for the requirement of a more specific structure. The fact that orotic acid has from 5 to 10 times the activity of ureidosuccinic acid and that both of these compounds are incorporated into the same 2 nucleotides(4) is evidence that orotic acid is a probable intermediate in the conversion of ureidosuccinic acid to nucleic acids. The postulated steps in the formation of orotic acid from ureidosuccinic acid are shown in Fig. 1.

Ureidofumaric acid is shown in Fig. 1 as a possible intermediate. That the double bond is formed before ring closure is evidenced by the fact that dihydro-orotic acid exhibits only 0.5% the activity of orotic acid. Attempts to prepare ureidofumaric acid in these laboratories have failed up to the present.

POSTULATED PATHWAY OF OROTIC ACID SYNTHESIS FROM UREIDOSUCCINIC ACID



A. UREIDOSUCCINIC ACID B. UREIDOFUMARIC ACID C. OROTIC ACID

FIG. 1.

Summary. A number of ureides were tested for orotic acid-like activity using *L. bulgaricus* 09. Ureidosuccinic acid, the only compound that could be visualized to yield a substituted pyrimidine ring by cyclization, was the only ureide with significant activity.

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Negative Effects of Oral Monobenzyl Ether of Hydroquinone in Malignant Melanoma in Man. (19456)

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Monobenzyl ether of hydroquinone ("agerite alba") is an effective inhibitor of melanin formation both *in vitro* and *in vivo*(1,2), perhaps due to direct action on tyrosinase. The compound has been described to cause occupational leukoderma(3). Prolonged oral administration produces depigmentation of the skin in pigs(4) and in guinea pigs(5).

Although melanin formation by malignant melanoma may not be intrinsically involved in the initiation or continued growth of the neoplasm, it was considered of interest to ascertain whether monobenzyl ether of hydroquinone would affect melanoma or the course of patients with this disease.

Methods. Monobenzyl ether of hydroquinone* was administered orally, 3 times a day, in capsules containing 0.1 g or in tablets of 0.5 g. The dose of the compound ranged from 0.3 to 27 g per day, and was given for 18 to 214 days. Eight patients with metastatic melanoma, verified by at least one relevant biopsy, were treated, Table I. Their ages ranged from 27 to 63. There were 2 premenopausal and one post-menopausal women in the group. All patients were of the white-skinned races. Estimations were made at frequent intervals of the size, consistency and appearance of the metastatic lesions. Urine specimens were examined weekly for melanin by the ferric chloride method. The patients were followed by weekly determinations of hemoglobin, red and white blood cell counts, as well as repeated determinations of such chemical blood constituents as protein, nonprotein nitrogen, bilirubin and electrolytes.

Results. No effect upon the growth of the neoplasms, or beneficial effects upon the patients, were observed. A definite reduction in the size of the subcutaneous mass was observed early in the course of therapy in Case 8, but within 12 weeks the mass enlarged steadily in size despite doubling the dose of the chemical. There was no depigmentation of the lesions. Five patients had melanin in the urine during the course of the disease. No consistent effect of monobenzyl ether of hydroquinone was noted upon the melaninuria.

TABLE I. Oral Monobenzyl Ether of Hydroquinone in Patients with Malignant Melanoma.

Case No.	Sex	Age	Dose/day, g	No. days treated	No. days between course	Total dose /course, g	Total No. days observed
1	♂	62	.2– .3	21	—	2.9	40
2	♂	47	.3–15	51	28	181	—
			5 –10	7	—	60	253
3	♂	63	10 –15	18	—	250	90*
4	♂	28	5 –10	36	25	240	—
			10 –27	6	—	118	142
5	♀	55	.5–10	46	23	247.5	—
			10	12	—	120	72
6	♀	27	.5–15	46	—	472.5	135
7	♀	33	.4–12.5	41	25	188.3	—
			7.5–20	40	—	592.6	120
8	♂	36	.5–12.5	151	22	385.2	—
			10 –25	63	—	1085	365

* Alive.

* Purchased from Goodrich Rubber Co.

Nausea and occasional vomiting occurred in five patients, with daily doses of over 10 g per day. No other reactions were noted, and no effects were observed clinically or biochemically upon the functions of the kidneys, liver, or hemapoietic tissues.

Seven of the 8 patients died after an expected course of the disease; Case 3 is still alive. Necropsy examination in seven cases failed to reveal histologic evidence of toxic effects or other changes attributable to the ingestion of the chemical.

Conclusion. Daily oral doses of 0.3 to 27 g of monobenzyl ether of hydroquinone, for total doses of 2.9 to 1490 g in 21 to 214 days, had no effect upon the growth of malignant melanoma in 8 patients. Ingestion of monobenzyl ether of hydroquinone produced no toxic effects other than transient nausea and vomiting when the daily dose exceeded 10 g.

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Bactericidal Action Mediated by Antibodies Specific for Heterologous Antigens Adsorbed to Bacterial Cells. (19457)

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Many gram negative bacteria are killed, and some are lysed, when they are exposed to complement and homologous antibody. It is generally believed that the antibody which mediates the killing of the bacterial cells by complement is specific for somatic antigens (1-3), though antibody specific for flagellar antigens has been held responsible for bactericidal serum activity against motile strains of *Proteus*(4).

The present communication, based on data obtained in the course of a more detailed investigation of qualitative and quantitative aspects of the bactericidal reaction,* is concerned with the site at which the antigen-antibody reaction responsible for cellular death occurs.

It is known that bacterial cells may adsorb to their surface components of the medium in which they are grown or suspended(5,6). The presence of some of these adsorbed substances can be demonstrated by the observation that such coated bacterial cells are agglutinated by antibody specific for the adsorbed substances. It appeared of particular interest to determine whether, in the presence of complement, reactions between antibody and adsorbed antigen would cause the death of susceptible bacteria. That bactericidal activity might actually occur as the result of such reactions was indicated by the work of Angerer and Hartoch(7) who demonstrated accelerated lysis of *Vibrio* cells which had first been exposed to suboptimal amounts of bactericidal horse serum and then to rabbit anti-horse serum. Bacterial antigens were chosen as the coating agents in this investigation because their use promised to throw some light on the quantitative aspects of the bactericidal reaction and also on the apparent lack of specificity in the bactericidal action of normal sera. These problems will be dealt with in subsequent papers.

Materials and methods. The antigens employed for the coating of bacteria were extracted with trichloroacetic acid as described by Boivin(8). They were precipitated by the addition of alcohol to a final concentration of

* Thesis submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy at Washington University.

75% by volume, washed repeatedly in 75% alcohol, and finally dried over calcium chloride under aseptic conditions. When required, sufficient amounts of dry antigen were weighed out to yield the desired volume of the 0.1% solution (in physiological saline) to be used in the coating procedure. In order to coat bacterial cells of one species with antigens derived from another species, the growth from three 18-hour slant cultures was harvested and washed twice with saline. The packed cells were then evenly suspended in 5 ml of the sterile 0.1% solution of antigen, and a similar amount of cells was suspended in saline, to serve as control. After 30 minutes of incubation at room temperature, with occasional agitation, both cell suspensions were diluted with saline until they contained approximately 5×10^4 viable cells per ml.† To test the *bactericidal activity* of immune sera against coated (test) and uncoated (control) bacteria, the method of Felix and Olitzki(1) was modified so that more nearly quantitative titrations could be obtained. Serial dilutions of inactivated immune serum, in 0.2 ml amounts, were distributed into a series of sterile tubes and, while the tubes were kept in an ice-water bath, the following reagents were added in quick succession: 0.2 ml of the standard inoculum of bacteria (approximately 10^4 viable cells), 10 minimum hemolytic units of complement (contained in 0.1 ml of a suitable dilution of specifically absorbed fresh guinea pig serum), and 0.5 ml of half-strength broth. After mixing of the contents the tubes were incubated at 37°C for 30 minutes, returned to the ice-water bath, and 2 ml of chilled, quarter-strength broth were added to each tube. Finally two 1 ml aliquots from each tube were transferred to Petri plates and mixed with proteose No. 3 agar at 45°C. Colonies present after 18 hours of incubation at 37°C were counted by the microscopic method described by Felix and Olitzki(1). *Controls* for the sterility of the various reagents, for lack of bactericidal activity of either the immune serum or of complement alone, and a control on the bacterial content of the inoculum (immune serum and complement omitted) were included in each test.

† Because the serial dilutions required in this step generally involved a 10000-fold dilution of the suspending fluid, it was not necessary to wash the cells free of unadsorbed antigen.

Application of Student's "t" test for statistical significance to a large number of data obtained in these and other bactericidal tests demonstrated that a difference of 15% between the number of colonies present on the inoculum control plates and the number of colonies arising from any given experimental mixture of immune serum, bacteria, and complement, was highly significant. Therefore, the titer of a serum was defined as the highest dilution of the serum which under the standard conditions of the experimental procedure caused the death of 15% or more of the inoculum. In order to *free the immune sera* used in these experiments and the guinea pig sera which were employed as complement of undesired antibodies, absorptions were carried out with massive doses of heat-killed, washed cells of the indicated bacterial strains. Absorptions of the guinea pig sera were carried out at 0-4°C, those of the immune sera at 37°C, for one hour, twice in succession with each absorbing organism. The *immune sera* were prepared by immunizing rabbits with 5 to 6 doses of bacteria. The bacterial cultures were obtained from the stock collections of the Department of Bacteriology and Immunology, Washington University, and the Department of Bacteriology, University of Kentucky.

Results. The data presented in Table I show that cells of each of the 3 *Salmonella* species tested (*S. münchen, S. potsdam* and *S. ballerup*) could be rendered sensitive to the bactericidal action of complement in the presence of an anti-*Escherichia coli* immune serum if they were first exposed to a solution of *E. coli* antigens. This bactericidal activity against coated bacteria was present even in high dilutions of the immune serum (1:640 to 1:20,480), while uncoated (control) cells were not killed by any of the serum dilutions tested.

It appeared of interest to determine, in a preliminary manner, whether cells of other gram negative bacterial species could also be conditioned to the killing action of comple-

TABLE I. Bactericidal Action Mediated by *Escherichia coli* Antiserum Against *Salmonella* Cells Which Have Been Coated with *E. coli* Antigens.

	Anti-*E. coli* serum absorbed with											
	S. münchen tested against *S. münchen*				*S. potsdam* tested against *S. potsdam*				*S. ballerup* tested against *S. ballerup*			
	Uncoated		Coated*		Uncoated		Coated*		Uncoated		Coated*	
Final dilution of *E. coli* antiserum	No. survived	% killed	No. survived	% killed	No. survived	% killed	No. survived	% killed	No. survived	% killed	No. survived	% killed
1:80	2790	0†	2210	35	2920	0	1750	36	3310	0	2370	34
1:320	2860	0	2290	32	2940	0	1860	32	3240	0	2610	28
1:1280	2750	0	2610	24	2880	0	2470	0	3210	0	3050	16
1:5120	2620	0	2650	22	2830	0	2680	0	3280	0	3230	0
1:20480	2660	0	2930	15			2660	0			3540	0
1:81920	2740	0	3010	0								
Controls												
Serum	2740		3360		2880		2750		3190		3580	
Complement	2780		3450		2880		2720		3220		3610	
Saline	2720		3460		2820		2700		3240		3550	
Titer	<1:80		1:20480		<1:80		1:640		<1:80		1:1280	

* Coated with antigens extracted from *E. coli*. † Less than 15% of inoculum killed.
Complement used was absorbed twice with *E. coli*, and twice with *S. münchen*, *S. potsdam*, or *S. ballerup*, respectively.

TABLE II. Bactericidal Action of Anti-*S. typhosa* O 901 Serum Against Cells of Unrelated Bacterial Species, Coated with *S. typhosa* O 901 Antigens.

	Anti-*S. typhosa* serum absorbed with											
	K. pneumoniae tested against *K. pneumoniae*				*S. marcescens* tested against *S. marcescens*				*P. morgani* tested against *P. morgani*			
	Uncoated		Coated*		Uncoated		Coated*		Uncoated		Coated*	
Final dilution of *S. typhosa* antiserum	No. survived	% killed	No. survived	% killed	No. survived	% killed	No. survived	% killed	No. survived	% killed	No. survived	% killed
1:40	n.t.†		n.t.†		2740	0‡	1750	35	3280	0	1680	27
1:80	3010	0	2950	0	2670	0	2090	22	3210	0	1920	17
1:160	2970	0	3010	0	2710	0	2740	0	3190	0	2310	0
1:640	2990	0	3070	0	2690	0	2670	0	3240	0	2400	0
1:1280	3020	0	3110	0	2630	0	2740	0	3180	0	2360	0
Controls												
Serum	2970		3180		2610		2740		3090		2300	
Complement	3030		3200		2570		2670		3160		2340	
Saline	3000		3160		2690		2630		3180		2370	
Titer	<1:80		<1:80		<1:40		1:80		<1:40		1:80	

* Coated with antigens extracted from *S. typhosa* O 901. † n.t. = not tested.
‡ Less than 15% of inoculum killed.
Complement used was absorbed twice with *S. typhosa* O 901, and twice with *K. pneumoniae*, *S. marcescens*, or *P. morgani*, respectively.

ment and of antibody specific for substances adsorbed to their surface. One strain each of *Klebsiella pneumoniae*, *Serratia marcescens*, and *Proteus morgani* was selected, and cells of these strains were exposed to a solution of antigens derived from *Salmonella typhosa*. The coated cells, as well as uncoated cells of the test strains, were then exposed to serial dilutions of an anti-*S. typhosa* serum which had been previously freed of natural antibodies against the test strains by suitable absorptions. The results, presented in Table II, show that both the *S. marcescens* cells and the *P. morgani* cells which had adsorbed antigens from *S. typhosa* became sensitive to the bactericidal action of complement in the presence of anti-*S. typhosa* serum, while the mucoid strain of *K. pneumoniae* apparently failed to acquire sensitivity to this serum.

Discussion and summary. The data presented support the view that the bactericidal reaction is mediated by the reaction of antibody (and complement) with antigens located at the surface of the bacterial cells. Since it

may be assumed, *a priori,* that sensitization to killing by complement requires attachment of certain minimum amounts of antibody to the cell, and this in turn depends on the presence of a sufficient number of specific antigen sites, the findings reported here may serve to explain the reported successful sensitization of a heavily flagellated *Proteus* strain by anti-flagellar antibody(4), and the consistent failure to observe sensitization of the less heavily flagellated *Salmonella* strains by antibody specific for their flagellar antigens.

The apparent differences in acquired sensitivity of the various test strains to the bactericidal effect of complement and of antibody specific for adsorbed antigens need not be considered significant. In some instances, such as in the case of the mucoid *Klebsiella* strain, resistance may be inherent in the strain (9), in other cases the composition of the cell surface may limit its capacity to adsorb the particular test antigen. Optimum conditions for adsorption were not extensively investigated.

It is believed that findings presented here should be taken into consideration in the interpretation of apparently non-specific bactericidal activities of sera. Furthermore, since the bactericidal test is an extremely sensitive method for the detection of small amounts of antibody, further refinement of the technic may lead to the development of methods suitable for the detection of trace amounts of antibody against antigens that may be adsorbed to suitable bacterial strains.

Conclusion. The bactericidal activity of complement is mediated by the reaction between antibody and antigens located at the surface of bacterial cells. These surface antigens need not be constituents of the cell but may be substances which have been artificially adsorbed to its surface.

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Effects of Exposure to Cold and of Dietary Restriction upon Globulin Nephritis in Rabbits.* (19458)

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Glomerulonephritis has been induced in both rats and rabbits by a variety of experimental procedures(1-9). These have included intravenous injection of heterologous serum proteins(1-4), anti-kidney antibodies(5-7), and extracts of ground kidney and streptococci(8,9). Most attempts to increase or decrease the severity of experimental nephritis have been made with so-called "nephrotoxic" nephritis, *i.e.,* that produced by injection of an anti-kidney serum from another species. This type of nephritis has been inhibited in rabbits by preliminary general exposure of the body to x-radiation(10). In rats nephrotoxic nephritis has been prevented by restriction of dietary protein(11).

A very severe acute glomerulonephritis has been induced in rabbits by massive intravenous injections of bovine serum gamma globulin(12-14). The present report deals with the

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effects of 2 additional factors upon the nephritis produced in this way. In the first experiment, it is shown that by exposing animals to a cold environment during the experimental period, this globulin-induced nephritis is increased in severity, and possibly in incidence. A second group of experiments indicate that nephritis is completely prevented by relatively minor reduction in the animals' food and water intake.

Materials and methods. Seventy-two albino rabbits of mixed breed were used. All were maintained in separate cages, fed Ogilvie Miracle Chow and given tap water. Unilateral nephrectomy was done 3-6 weeks before further treatment. The animals of Groups I-IV inclusive were injected intravenously on 2 occasions 10 or 11 days apart with 1 g/kg body weight of purified bovine serum gamma globulin given as a 10% aqueous solution in 0.95% NaCl. The second injection was preceded by a desensitizing intravenous injection of 5-10 cc of a 1% globulin solution. During the experimental period blood was drawn at various times from animals of all groups for precipitin tests. Urine was collected daily from individual animals of all groups via a screen grid and metal funnel. Twenty-four hour urine output was measured and daily analysis for protein content was made as well as a microscopic search for red blood cells and casts. Animals were killed 6 or 7 days after the second globulin injection and autopsies performed immediately. Tissues were fixed in Zenker-formol and microscopic examination made of heart, kidney, spleen, thymus, lung, and adrenals. The animals were divided into the following groups:

Group I—Twenty-four animals were kept at room temperature and allowed *ad lib.* food and water intake. They received 2 massive globulin injections as outlined above. *Group II*—Sixteen partly shaved animals were placed in a refrigerated room where the temperature fluctuated between —9.4°C and +4.4°C. After 3 days of exposure to low temperature these animals were injected with bovine serum gamma globulin, exactly as those of Group I. Apart from continuous exposure to cold, animals of this group were treated in exactly the same manner as those of Group I. *Group III*—Six animals, kept at room temperature, were treated in the same manner as Group I, except that each was restricted to 70 g of food and 130 cc of water per day. *Group IV*—Six animals, kept in the refrigerator and on the same restricted diet as those of Group III, were injected with bovine gamma globulin in the usual way. *Groups V and VI* consisted of 10 animals each, serving as controls for Groups I and II. Group V was kept on *ad lib.* diet at room temperature and subjected to the same procedures (bleeding, urinalysis) as Group I. Group VI was handled similarly but was housed in the refrigerated room throughout the experimental period. The animals of Groups V and VI received no injections of bovine gamma globulin.

Results. Diffuse glomerulonephritis developed in globulin-treated animals on *ad lib.* diet, whether kept at room temperature or in the cold (Groups I and II). The severity of the disease was graded roughly as zero to 4 plus (13). The incidence of lesions of the various grades in these 2 groups of animals is shown in Table I. It is seen that whereas 75% of the animals of Group I developed diffuse nephritis, similar renal damage was found in *all* animals treated in the cold (Group II). It was further found that severe (+++ to ++++) nephritis occurred in 29% of Group I animals, while 56% of animals of Group II had nephritis of this degree.

There was no difference in the gross or microscopic features of the renal lesions in these 2 groups. The remaining kidney was usually larger in nephritic animals than in controls kept at the same temperature. The mean kidney weight for room temperature control animals (Group V) was 12.4 g, the largest being 16.0 g, compared to an average of 13.8 g in cold room controls (Group VI). Group I animals had an average kidney weight of 14.3 g, whereas those of Group II had kidneys averaging 17.8 g. Kidneys in which severe nephritis was present were usually distinctly yellower and paler than normal, but not hemorrhagic. Microscopically these kidneys showed diffuse damage to all glomeruli similar to that reported previously(13). The lesions corresponded in appearance to those of

TABLE I. Effect of Exposure to Cold on Incidence and Severity of Experimental Glomerulonephritis.

Group	No. of animals	Severity of nephritis 0	+	2+	3+	4+	Incidence % with nephritis	% with severe nephritis
I. Room temp.	24	6	4	7	4	3	75 (60.2–86.3)*	29 (17–44.1)
II. Refrigerated	16	0	2	5	7	2	100 (86.6–100)	56 (37.5–73.7)

* Figures in parentheses represent confidence limits (%) at probability of .10.

the extra-capillary glomerulonephritis of Fahr (15), or Type I nephritis of Ellis(16).

Urinary changes of acute nephritis (proteinuria, cylindruria, hematuria) generally became manifest between 7 and 10 days after the initial globulin injection. The urinary changes in the animals treated at room temperature were less severe after the 10th day, but in animals treated in the cold, there was a secondary exacerbation between the third and sixth days after the second massive injection with globulin. Uremia developed in 3 of the 40 treated animals and one died in uremia.

Serum protein estimations on several of the animals showed a rise in globulin, and fall in albumin, coincident with the development of nephritis, and also at about the time when antibodies to the injected globulin became demonstrable in the sera. The details of these findings, together with more extensive studies in similar experiments will be published(14).

The findings in animals of Groups III and IV were in striking contrast to those in the first 2 groups. In none of these animals was there any morphological evidence of glomerulonephritis, nor did any of them show persistent proteinuria during life.

Table II shows relative daily weight gain in the various groups. It is evident that animals of Group III gained only about half as much per day as animals on *ad lib.* diet, while those of Group IV registered only very slight weight gain.

The results of ring tests to demonstrate precipitins to bovine gamma globulin are shown in Table III. It is evident that all globulin-treated animals on *ad lib.* diet had precipitins on the 10th day, whereas only 5 of the 6 in Group III and one of the 6 in Group IV gave positive reactions at this time.

Discussion. There seems little doubt that the diffuse glomerulonephritis which appeared in animals of Groups I and II was in fact produced by the bovine gamma globulin injections they received. Similar lesions were not found in any of the unilaterally nephrectomized control animals (Groups V and VI), kept under identical conditions, nor were such lesions found in any of the kidneys surgically removed before treatment. The numbers of animals in the first 2 groups are too small for the difference in incidence of nephritis to be significant. However, within confidence limits of 0.005, the increased percentage of refrigerated animals showing severe nephritis is statistically significant.

It should be emphasized that grading of incidence and severity was done before any

TABLE II. Effect of Dietary Restriction on Rate of Weight Gain and Development of Glomerulonephritis.

Group	No. of animals	Avg wt when killed, kg	Avg daily wt gain, g	No. with glomerulonephritis
I	6	2.66	25.2	4
II	15	2.33	25.2	15
III	6	2.12	13.6	0
IV	6	1.74	2.5	0
V	10	2.81	25.8	0
VI	10	2.60	26.2	0

TABLE III. Effect of Dietary Restriction on Antibody Production. 6 animals in each group.

Group	Day 0	Day 10	Glomerulonephritis
I	0*	6*	4
II	0	6	6
III	0	5	0
IV	0	1	0

* Figures indicate number of animals showing precipitins to bovine serum gamma globulin.

statistical calculations had been made. Furthermore, in the grading criteria employed, the difference between severe (+++ and ++++) and mild (+ and ++) lesions was so striking as to render incorrect classification in these categories very unlikely.

The findings in animals of Groups III and IV indicate complete inhibition of experimental globulin nephritis, apparently produced by minor general dietary restriction. Since all animals were otherwise treated in exactly the same manner, this suggests that inhibition was in fact the result of dietary restriction.

We have suggested elsewhere(14) that the development of experimental glomerulonephritis may be a function of quantitative antibody response, *i.e.*, the greater the amount of antibody produced, or the faster the rate of its production, the greater the liability to nephritis. The present findings are in agreement with this suggestion. While accurate measurements of food intake were not made on rabbits of Group II, it was noted that larger quantities of food were required to maintain their rate of weight gain. Furthermore, refrigerated animals on restricted diet gained much less weight than animals maintained at room temperature on the same diet. These findings seem to indicate a higher rate of metabolic turnover in the refrigerated animals. Other investigators(17) have found that antibody protein participates in the general metabolic ebb-and-flow to the same extent, and at about the same rate, as do other tissue and body proteins. It has also been reported that antibody production is increased in animals exposed to cold(18,19). It is possible, therefore, that the increased severity of nephritis in animals exposed to cold was related to a general increase in metabolic turnover, in which increased antibody production was the determining factor. On the other hand, prevention of nephritis in animals on restricted diet appears to have been accompanied by delayed antibody production. This is particularly noticeable in the refrigerated animals (Group IV).

It is clear that further investigation will be required before much more can be said of the mechanism of the dietary inhibition of nephritis. The fact that antibodies did appear in some of the animals on restricted diet is in keeping with the suggestion that the development of nephritis may be related to the potency of the antibody reaction rather than to its presence or absence.

It should be emphasized that any experimental procedure which impaired the animals' appetites might produce a similar result and receive unjustified credit as a preventive. Indeed, certain experimental results reported in the past might profitably be reassessed in the light of this finding.

Summary. 1. Eighteen (75%) of 24 unilaterally nephrectomized albino rabbits given 2 massive injections of bovine serum gamma globulin at 10 to 12 day intervals, developed lesions of acute diffuse extra-capillary glomerulonephritis. The disease was severe in 7 (29%). In 16 animals similarly treated, while continuously exposed to a cold environment, nephritis developed in 100%, and was severe in 56%. It is concluded that these differences in severity are probably significant, and may be related to a general increase in metabolic activity, in which increased antibody production is the important factor. 2. None of 12 animals similarly treated while under mild starvation contracted nephritis whether maintained at room temperature or exposed to cold. Inhibition of nephritis appeared to be accompanied by impairment of antibody production. 3. In view of these results it is suggested that the dietary status of animals in similar experiments must be evaluated before credit can be given any other therapeutic agent for prevention or inhibition of experimental nephritis.

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Experiments with C^{14}-Menadione (Vitamin K_3).* (19459)

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Dam(1) established that hemorrhage in chickens on certain diets was due to the absence of a new accessory food factor, vit. K. Schonheyder(2) established that the hemorrhagic condition was due to a decrease in plasma prothrombin, and Greaves(3), following up the work of Hawkins and Brinkhous (4), established that the prothrombin deficiency in obstructive jaundice was due to failure to absorb vit. K, the presence of bile in the intestine being necessary for this. Extensive experimental and clinical observations (5-7) have been made connecting a decrease in plasma prothrombin concentration with interference with liver function. By inference, it has been assumed that vit. K acts through the liver, although little evidence has been obtained for this. Likewise little information is available regarding distribution of the vitamin in tissues. The senior authors have previously reported the results of studies of dicumarol labelled with C^{14}(8). As this substance and vit. K appear to be related as antivitamin and vitamin, radioactive K has been synthesized as 2:C^{14}-methyl-1:4-naphthoquinone(radioactive menadione) and administered to mice and dogs. The present communication reports the results of these experiments.

Methods. Experimental procedure. 2-methyl-1:4-naphthoquinone with C^{14} in the methyl group was synthesized by Mr. R. V. Phillips, as described elsewhere(9). The specific activity was 4.10 x 10^4 counts per minute per mg (c.p.m./mg). The material was dissolved in corn oil and injected intramuscularly into dogs and mice. The mice were sacrificed at different time intervals and the activity determined on aliquots of the dried tissues. To determine the activity of the dog tissues, the organs were boiled in dilute alkali and an aliquot counted. Radioactivity measurements were made using a gas-flow type of Geiger-Müller Counter. The efficiency of the counting system was checked frequently with a sample of C^{14}-dicumarol used as a standard. Counts were corrected for background, efficiency change with time, self-absorption, resolving time and geometry. Twice the standard deviation of the background was 9.6

TABLE I. Test of Radioactivity of Expired CO_2 of Mouse after Receiving C^{14}-Menadione.

Sample hr	Wt of $BaCO_3$ counted, mg	Observed activity –bkgd. c.p.m.	Total activity
0-11	11	2.6	
[illegible]	[illegible]	–	N.A.
17-24	60	3.8	

N.A. = No significant activity.

* Assisted by grants from the National Research Council of Canada to L. B. Jaques and J. W. T. Spinks.

c.p.m. and a count greater than background by this amount was considered significant. Values differing from background by less than this are reported as no activity (N.A.). A depletion of vit. K in dogs was effected by a cholecystonephrostomy according to the Kapsinow-Markowitz(10,11) technic, anastamosing the gall bladder to the right kidney. A total of 6 dogs were prepared in this way. A weekly check was made for the detection of bile in the feces and urine to ensure that the cholecystonephrostomy was complete. After death, the animal was checked at autopsy to make sure that the fistula was patent and that there were no other channels for bile. It took from one to 3 months after the cholecystonephrostomy for the prothrombin time to increase to double its normal value. Prothrombin times were determined by the usual method using 0.02 M calcium chloride solution and a commercial rabbit-brain powder supplied by Cappel Laboratories, Wayne, Pa.

Experiments with normal mice. Expired CO_2. To check the possibility of the radioactive methyl group being metabolized to CO_2, a 27 g mouse was injected with 1.0 mg of vit. K_3, placed in a metabolism cage for 24 hours and the expired CO_2 collected from the mouse. The results are shown in Table I.

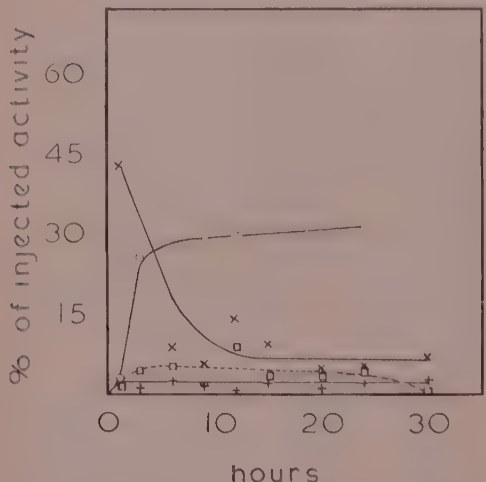

FIG. 1. Radioactivity in mice after inj. of C^{14}-vit. K_3. 1 mg/mouse intramusc. into right hind-leg ×——× inj. site; ○——○ urine; □——□ gastrointestinal tract and contents; +——+ blood.

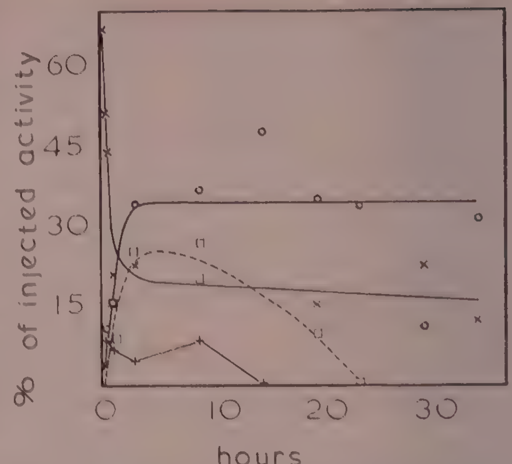

FIG. 2. Radioactivity in mice after inj. of C^{14}-vit. K_3. .1 mg/mouse intramusc. into right hind-leg. ×——× inj. site; ○——○ urine; □——□ gastrointestinal tract and contents; +——+ blood.

It can be seen that there is no activity in the expired CO_2 from a mouse, following C^{14}-vit. K_3 administration.

Tissues. Two series of mice were injected with the labelled vitamin and then sacrificed in succession at varying times. Twelve mice received 0.1 mg of vit. K and eleven received 1.0 mg of the vitamin. Excreta were collected up to the time of sacrifice at which time, tissues and organs were removed. All samples were tested for radioactivity. The results are shown for blood and excreta in Fig. 1 and 2 and for tissues in Table II. The vitamin was injected intramuscularly into the right hindleg and the latter was counted separately and reported as the activity of the injection site. In the case of the zero sample, the animal was sacrificed and the K then injected. 94% of the activity was recovered from the injection site of this animal. It can be seen that the activity disappeared rapidly from the injection site in the first hour after the injection, leaving a small amount of residual activity in the depot. This rapid loss of activity from the injection site was accompanied by the appearance of activity in the blood. The total amount of activity in the blood was calculated on the assumption that the weight of the total blood volume was 10% of the body weight. With the larger dose, the ac-

TABLE II. Radioactivity of Tissue of Mice after Injection of C^{14}-Menadione.

Time after inj.	Liver		Lung		Kidney		Spleen		Muscle		Carcass	
	Dose, mg											
	.1	1	.1	1	.1	1	.1	1	.1	1	.1	1
0	*	—	*	—	*	—	*	—	—	—	*	—
5 min	"	.42	"	.03	"	.34	"	—	—	—	"	—
15	"	*	"	.06	.88	.56	"	—	—	—	"	—
30	"	"	.58	*	.83	*	"	—	—	—	"	—
					.85							
60	"	.23	"	—	.75	.34	"	—	—	*	"	2.24
		.34	1.17	—	*	2.34	—	*	—	"	—	*
3 hr	"	*	.49	—	"	.17	*	"	—	"	*	"
			*									
6	—	.60	—	—	—	.26	—	.04	—	4.48	—	5.72
9	*	.45	*	—	*	*	*	*	—	*	*	*
			.21									
12	—	1.31	—	—	—	.49	—	"	—	"	—	"
15	*	.32	.36	—	*	.15	*	"	—	"	*	2.75
20	"	*	*	*	"	.66	"	"	—	—	"	*
24	"	"	"	.04	"	.30	.85	"	—	—	"	"
30	"	"	"	*	—	*	*	"	—	—	"	"

All values given as % of injected dose; 1 mg of C^{14}-menadione = 4.10×10^4 counts/min. Mice 18-26 g weight.

* No significant activity (<9.6 c.p.m./sample).

tivity per mg of dry sample and consequently the total blood activity was approximately twice that with the smaller dose, and the activity could be detected for a longer period of time. 0.3% of the larger dose, and 3% of the smaller, distributed in the mouse total blood volume would be detectable. Relatively little activity was found in tissues. Liver, lung and kidney occasionally showed small amounts of activity. However, the values were just within the limits of significance and accounted for only 1% of the activity injected. Skin, bone and muscle of some mice were treated separately with no significant activity showing. The results are included under carcass. The activity in the kidney may be explained as due to activity in glomerular filtrate and urine, since values of the same order were obtained on urine taken directly from the bladder. Activity in lung was observed in an irregular manner, in liver only with the 1 mg dose. In the latter case for the eight mice sacrificed between 0 and 12 hours, six had livers which gave counts more than 12.8 c.p.m. above background (significant at the 0.05 level) and the average activity in the liver was equivalent to 5 μg of vit. K. 30-40% of the activity injected was recovered in the urine. This excretion occurred largely in the first 3 hours after the injection. No activity was found in the feces. The gastrointestinal contents showed some activity reaching a peak 3 hours after the injection for the smaller dose. The gall bladder and contents showed traces of activity coincident with that in the gastrointestinal tract.

Blood. In view of the small residual amount of radioactivity which appeared to persist in blood, in a further experiment a fractionation of the blood was attempted, both to concentrate the activity and thus get a more accurate count, and to determine the fraction of the blood containing the activity. The fractionation procedure followed was that of Cohn *et al.* (12). Fourteen mice were injected with a total of 12 mg of vit. K_3. These were sacrificed at the end of 15 hours and the blood collected by cardiac puncture. A total of 6.0 cc of blood was obtained from the 14 animals. The blood was drawn with a silicone-coated syringe and placed in a silicone-coated graduated centrifuge tube where it was diluted with 0.5 cc of 3.8% sodium citrate solution. The blood was immediately transferred to the cold room at −5°C for fractionation. After removal of 0.5 cc of blood for counting, the remainder was centrifuged at high speed. The plasma and white cells were drawn off separately and the red cells washed twice with 2.0 cc of isotonic saline. The wash from the red cells was

TABLE III. Radioactivity of Various Fractions from Pooled Blood of 14 Mice 15 Hr after Injection of C^{14}-Vit. K.

Fraction	Observed activity (minus background) c.p.m.	Total activity, c.p.m. × 10^3	% total injected activity
Whole blood	10	1.40	.29
Hemoglobin	11	1.15	.23
Washed r.b.c.	11	1.05	.21
W.b.c. and platelets	9.6	.051	.01
Wash from r.b.c. and platelets	33	.282	.06
Ghosts	5.1		*
Albumin, V.	22	.244	.05
R-globulin, II	4.5		*
Prothrombin, III-1,2	2.6		"
Fibrinogen, I. III-3	5.1		"
Cholesterol, III-0	1.9		"
Cholinesterase, IV	5.1		"
Small albumins, VI	1.9	.109	.02

* No significant activity.

added to the platelets and w.b.c. and centrifuged. The plasma was poured into a 50 ml plastic centrifuge tube and precipitated with ethanol, the fractions being prepared as described by Cohn *et al.* The counts on the various fractions are shown in Table III. Significant activity was found in whole blood, hemoglobin, washed red blood corpuscles, white cells and platelets, the wash from the r.b.c.'s and platetlets, and the albumin-fraction. The albumin (fraction V and VI) and the wash (from r.b.c.'s platelets) alone showed a sufficiently high-specific activity to give reliable estimates of activity. With the relatively gross errors in the estimations of activity on whole blood and hemoglobin, due to low specific activity, it is difficult to estimate how much radioactivity is associated with the latter but in as much as the wash from the r.b.c.'s had a higher specific activity than the albumin fractions and an equal total activity, this indicates that the r.b.c.'s showed a higher total activity than the albumins. There was definitely no activity associated with the fibrinogen, prothrombin or globulin fractions.

Urinary products. In order to examine the nature of the radioactive urinary products, urine was subjected to paper chromatography. Fresh urine was used from a mouse which was injected with 2 mg of vit. K_3. Two drops of this urine placed on a one inch square of filter paper registered 1.92 x 10^3 c.p.m. The solvent used was made up of 3 parts butanol to 1 part methanol and saturated with water, to which was added a few drops of acetic acid. Two drops of the urine was placed on one end of a filter paper strip one inch in width. These strips were suspended in beakers containing the solvent mixture and were developed for 30 hours inside a large covered glass cylinder. At the end of 30 hours the strips were dried and cut in 1 cm lengths for counting. Fig. 3 shows the location of the radioactivity on the strip. A radioactive component with an R_F value of 0.41 is present. Two drops of 2 N acetic acid were added to 4 drops of urine and the sample was subjected to hydrolysis by heating at 100°C for 2 minutes. When a chromatogram was developed in the same way a second component (R_F = 0.25) was observed along with a decrease in the amount of the first component. This second component has also been obtained consistently with urine that has stood some days, suggesting that this compound can be formed also by bacterial action.

Experiments on cholecystonephrostomized dogs. Vit. K-deficient dogs were produced by performing a cholecystonephrostomy one to 3 months prior to the experiment. At the time of the experiment, the animals had a prothrombin time of 11-20 seconds (normal—8.2 seconds) and all showed a very pronounced

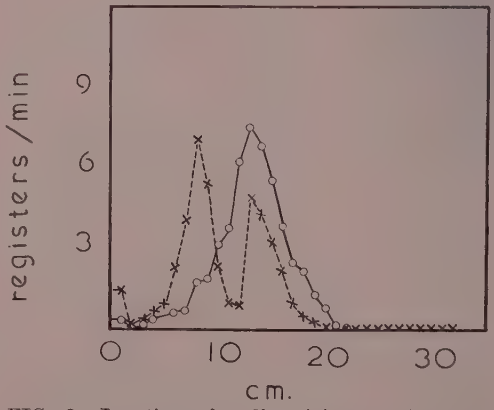

FIG. 3. Location of radioactivity on chromatograms of urine after inj. of C^{14}-vit. K_3. Mouse received 2 mg of C^{14}-vit. K intramusc. into right hind-leg. ○——○ fresh urine; ×——× urine after heating in .5 N acetic acid. 1 register = 64 counts.

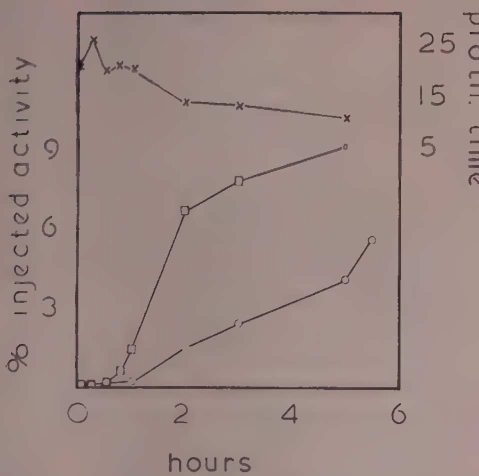

FIG. 4. Excretion of radioactivity and prothrombin time of cholecystnephrostomized dog. 1 mg/kg C^{14}-vit. K_3 inj. intramusc. at time zero. ×——× prothrombin time in sec.; □——□ urine from left kidney only; ○——○ bile.

clinical tendency to hemorrhage. The animals were anesthetized with nembutal, the left ureter cannulated to collect urine and the gallbladder to collect bile. Hemorrhage from this procedure occurred and was controlled with topical thrombin, but the animals died in 5 hours. One mg of radioactive vit. K per kg body weight was injected intramuscularly into the right hind-leg. Blood, urine, and bile samples were taken at intervals for determinations of radioactivity and aliquots of all tissues were taken at autopsy. The results of one experiment are shown in Fig. 4. The dog weighed 6.8 kg and had a prothrombin time of 19.4 seconds. 6.43 mg of C^{14}-vit. K_3 were injected intramuscularly into the right leg. Samples of blood, urine and bile were withdrawn simultaneously at 1, 5, 15, 30, 45 and 60 minutes, then at the 2-, 3- and 5-hour intervals after the injection. 0.2 ml of blood was used for counting each time. No activity was found in the blood. The prothrombin time and excretion of radioactivity in the urine and bile are shown in Fig. 4. The dog died 5 hours after the injection. On death, tissues were removed for measurement of radioactivity. The liver, blood, bone marrow, heart, spleen and kidneys showed no activity. The intestines with contents contained 16.4%, the site of injection 30.4% and the lungs 3.2%. Total activity of the urine samples collected from the left kidney was 9.0%, from the urinary bladder at the end of the experiment (right kidney) 5.0% and in the bile was 6.0% of the injected dose so that 70% of the activity injected was recovered. Urinary excretion of activity began thirty minutes after the injection and was at a maximum between the 1st and 2nd hour. The rate of excretion of activity in the urine in Fig. 4 is somewhat difficult to interpret as it must be noted that this is only for one kidney. Also there is the possibility that this kidney may be hyperactive with regard to urine secretion in order to compensate for the atrophied condition of the right kidney. However, the right kidney was found to be at least partially active as shown by the presence of activity in the urine collected from the bladder at the termination of the experiment.

In view of the smaller dose of labelled vit. K (1 mg/kg) given to the dog compared to the mouse, it would not be possible to detect activity in any tissues in this experiment unless a marked accumulation and concentration of activity had occurred. For instance, if the 2.6×10^5 c.p.m. were uniformly distributed in the total blood volume, there should be 388 c.p.m./ml of blood or 77 c.p.m. per sample counted. Hence it was necessary for over 10% of the injected dose to be present in the blood for it to be detected, and similarly 3% of the dose in the liver. However, with these limitations, no activity was present in the blood or liver. In contrast, a just significant amount of activity was present in the lungs. A second animal gave identical results except that no significant activity was found in the lungs.

Discussion. The highest dose, 1 mg per mouse, is as high as can be used safely if pharmacological effects are to be avoided. In a 20 g mouse this dose is equivalent to 50 mg/kg body weight which is the LD_{10} by the intraperitoneal route(14).

There is a very rapid absorption of the vitamin on intramuscular injection in both mice and dogs. One dog had no activity left at the site of injection within 5 hours, where-

as the other dog still had 30% present at the end of 5 hours. In the mice experiments it was found that 80% had been absorbed by the end of 4 hours. After the eighth hour there appeared to be very little further absorption, with activity still remaining after 35 hours. No pathological studies were made of the site of injection. However, Lord, Andrus and Moore(13) reported that no toxicity was evident at the site of intramuscular injections of menadione in corn oil in rats. The rapid rate of absorption of the vitamin from the site of injection in the mice is reflected in a rapid rise in blood activity. At each dosage level the amount of activity in the blood rose within 10 minutes to a maximal value of about 6.3 and 1.6% respectively and this level was maintained for hours.

A cholecystonephrostomy appeared to be effective in establishing a depletion of vit. K in dogs as evidenced by the very profuse hemorrhage on laparotomy. Depending upon individual variation between animals it took from one to 3 months to increase the prothrombin time to double its normal value. From the results reported it may be seen that the prothrombin time was back close to the normal value within 5 hours after the injection of radioactive vit. K. Andrus and Lord(15), working with rats, found that as little as 2 mg of menadione injected intramuscularly restored the plasma prothrombin by as much as 48% and the effect was evident as early as 8 hours after the injection. Garnett (16) found that a single intramuscular injection of vit. K in chicks with a prolonged blood coagulation time due to a diet deficient in vit. K, reduced the time to normal within one hour with a minimum effective dose of 0.135-0.27 mg in 3-week-old chicks weighing about 100 g.

Only in the mice receiving 1 mg (circa 40 mg/kg) was any activity found in the liver. An activity amounting to about 0.4% was present up to 15 hours after which time no further activity could be found. No activity was found in any of the dog livers. This observation shows quite definitely that the liver does not concentrate vit. K in contrast to dicumarol. Jaques and Spinks found that 20% of injected C^{14} dicumarol could be recovered from the liver. The activity of 10-20% of an equivalent injection of vit. K (0.25 mg) should have given 10^3-2×10^3 c.p.m. Actually, 1.0 mg of vit. K was administered and 20% (200 μg) would have an activity of 8.2×10^3 c.p.m. Canessa(7) gives values for the concentration in normal liver in different species equivalent to 20-250 μg of menadione per 100 g of tissue. A trace of activity was found in kidney and occasionally in lung and these gave actually higher specific activities than liver. The appearance of activity in the bile of the dogs indicates that a portion of the activity is excreted via this route. This is also indicated by the rapid appearance of activity in gall bladder and gastrointestinal tract in the mice. However, since it does not appear in the feces, this must be reabsorbed in the lower intestinal tract to explain the rapid fall in radioactivity of the gastrointestinal contents reported in Fig. 2.

From time to time, it has been postulated that vit. K serves as a prosthetic group of prothrombin, in which case the radioactive carbon should be recovered from prothrombin, after time has been allowed for synthesis to occur. However, the residual activity which appeared fixed in the blood was not attached to prothrombin. The albumin was the only fraction of the plasma proteins with which activity was associated. It had an activity of 0.07%. Fieser(17) found 75% of the vitamin in blood, bound to the albumin fraction. As shown in Table III, a larger quantity (0.23%) was found associated with the red cells. As no activity could be detected associated with the ghosts on hemolysis the activity must either be adsorbed on the red cells or combined with hemoglobin.

It was found in the mice experiments that there is a very rapid elimination of activity by the kidneys. Maximum excretion (totalling about 30%) is attained within 3 hours and no further increase was noted even after 24 hours. Urine samples from the bladder of 15-, 20- and 30-hour mice showed only 1% activity present and this is a further indication that after the first 3 hours, there is no further significant elimination by the kidneys. This maximal value of excreted activity of 30% corresponds with Richert's(18) observa-

tions on rabbits that 31-42% of a quinone was excreted in the urine after menadione administration, although he later reported that oxidative hydrolysis with ceric sulfate yielded quinone values 30% higher than by the previous hydrochloric acid procedure. Richert identified the product in the urine as 4-hydroxy-2-methyl-1-naphthyl sulfate. Doisy(19) has reported that when menadione is administered to rats 13-20% is excreted in the urine of which a part appears to be a β-glucuronide. Although no colorimetric analysis for the presence of a quinone was carried out, it may be gathered that it is excreted in a conjugated form. Richert found it necessary to hydrolyse the urine to obtain the color reaction and in the chromatographic analyses which were carried out on the active urine, the activity was found to undergo hydrolysis very readily giving rise to two compounds containing activity. Further, from the chromatographic work it was also seen that such hydrolysis would occur spontaneously due to bacterial action, so that evidence for the excretion of only one compound containing activity would be found only with fresh urine samples.

It was found in both normal and cholecystonephrostomized animals that there was no concentration of vit. K by the liver. This is in direct contradiction to the previous widely accepted belief that vit. K goes to the liver. Andrus and Lord(15) showed there was some antihemorrhagic factor present in liver. Since vit. K is not stored in the liver, an alternative hypothesis is that vit. K is material for prothrombin synthesis. If so, one would expect to find some activity in the prothrombin moiety itself since the liver is the site of formation of most of the plasma proteins. However, this was not the case. It is also to be noted that there was no activity in the dog bone marrow, although it is believed that some prothrombin formation may take place there also(5). The only alternatives left are (i) that the labelled methyl group does not participate in the physiological action of vit. K or (ii) that as no storage occurs, vit. K activity is dependent directly on blood levels of the vitamin. The first alternative seems very unlikely in view of the fact that only compounds with the methyl group are active, although experiments with vit. K labelled in some other position would provide more direct evidence. A marked dependence of prothrombin level on vit. K absorption has been shown by Mann *et al.*(20). With drainage of all abdominal lymph, a marked fall in the blood prothrombin level resulted within 18 hours. If we assume that vit. K is the only factor affecting prothrombin removed in this latter experiment it indicates a very marked dependence indeed on blood levels.

Summary. C^{14}-menadione (2-methyl-C^{14}-1:4-naphthoquinone) was prepared and administered intramuscularly to mice in doses of 1 and 0.1 mg, and to cholecystonephrostomized dogs in a dose of 1 mg/kg. The radioactivity was rapidly absorbed from the injection site and excreted in the urine, with only traces being detected in the blood. Quantities of radioactive material were not stored in any tissue. With the largest dose (1 mg/25 g mouse) a trace of activity was found in the liver and lung. The activity in the urine was attached to a single compound but a second component appeared on mild hydrolysis of the urine.

We are greatly indebted to Dr. J. M. Pepper for supervising attempts of one of us (P.S.) to synthesize C^{14}-menadione, to Miss Erica Lepp, who performed the prothrombin times, to Miss R. Keeler, who supervised the operating technics, to Mr. D. Wilson for assisting with the radioactivity measurements, to Dr. G. J. Millar for the plasma protein fractionation, to Dr. James Campbell for instruction in catherization technics.

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Effects of Intravenous Administration of Dextran on Renal Function.*† (19460)

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Expansion of the plasma volume produced by the intravenous infusion of human albumin and plasma is usually associated with an increased rate of renal plasma flow and occasionally with increased rates of glomerular filtration(1-3). Since it has been demonstrated that partially hydrolyzed dextran is an effective plasma volume expander(4-6), a study has been made of the effects of the intravenous administration of this substance on various renal functions.

Methods. Thirteen World War II male veterans, ages 27 to 34, who were normotensive, afrebrile, and well hydrated and who had no evidence of renal disease, were studied at rest and in the fasting state. Since the presence of large amounts of dextran interferes with the determination of inulin in plasma and urine, glomerular filtration rate was measured by the endogenous creatinine clearance, using Hare's creatinine method(7). Para-aminohippurate clearance (C_{PAH}) and measurements of tubular excretory mass (Tm_{PAH}) were performed as described by Goldring and Chasis(8), including bladder catheterization. Hematocrit determinations were done according to Wintrobe. In subjects 1-7 the procedure was as follows: After 2 control clearance periods, 500 cc of 6% dextran in normal saline‡ were given intravenously at the rate of 25 cc/min. Eight to 45 minutes after the termination of the infusion, 2 or more clearance periods were obtained. Subjects 8 and 9 received 1000 cc of 6% dextran; patient 10 was given 1500 cc; repeat clearances were begun immediately after the infusion was completed. In subjects 11-13, control clearances and Tm_{PAH} determinations were done on the first day. The following day 1000 cc of 6% dextran were administered intravenously, and the above procedures were then repeated 45 minutes to 2 hours following infusion. In 2 additional subjects a catheter was placed in the renal vein and simultaneous arterial and renal venous samples obtained for determination of PAH extraction before and immediately after the injection of 1000 cc of 6% dextran. All figures for clearances and Tm_{PAH} in the tables have been converted to a body surface area of 1.73 sq.m.

* Reviewed in the Veterans Administration and published with approval of the Chief Medical Director. Statements and conclusions of the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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‡ "Expandex," supplied by Commercial Solvents Corp., Terre Haute, Ind.

TABLE I. Studies with 500-1500 cc 6% Dextran.

Pt.	Amt dextran (cc)	Creatinine clearance Before	After	PAH clearance Before	After	Filtration fraction Before	After
1	500	132	146	508	588	.26	.25
2		106	132	565	722	.19	.18
3		122	128	559	704	.22	.18
4		94	102	455	472	.21	.22
5		126	155	640	829	.20	.19
6		156	162	675	701	.23	.23
7		157	166	777	732	.20	.23
8	1000	121	111	489	435	.25	.25
9		113	118	360	456	.31	.26
10	1500	193	204	973	859	.20	.24

TABLE II. Studies with 1000 cc 6% Dextran (24-48 Hr Between Before and After).

Pt.	Creatinine clearance Before	After	PAH clearance Before	After	Filtration fraction Before	After	Tm PAH (mg/min) Before	After
11	113	105	525	523	.22	.20	85	84
12	131	124	623	593	.21	.21	71	78
13	114	106	600	410	.19	.26	70	79

Results. Results of the administration of 500-1500 cc of dextran are presented in Table I. Creatinine clearance increased in all but one of the subjects, but the increase, based on previous experience, was considered significant only in subjects 2 and 5. PAH clearance increased in 3 subjects (Subjects 2, 3, and 5), changed very little in 4, and decreased in subjects 7, 8, and 10. Calculations of renal whole blood flow from C_{PAH} and hematocrit determination revealed increases of 103 cc/min. and 252 cc/min. in subjects 3 and 5, respectively, and a decrease of 43 cc/min. in subject 4. In subject 10, who received 1500 cc of dextran, the hematocrit fell from 41 to 32, and the renal blood flow decreased from 1650 to 1141 cc/min. From Table II it may be seen that with the exception of a decrease in C_{PAH} in subject 13, C_{Cr}, C_{PAH}, and Tm_{PAH} were not affected by the administration of 1000 cc of dextran.

Since a decreased renal extraction of PAH has been found after the rapid infusion of human serum albumin(1), this was measured in 2 additional subjects and found to be 89% and 92% before and 88% and 91% after dextran.

Discussion. The dextran solution used in these studies was essentially isosmotic with plasma, and previous work(4-6,9) has shown that the increases in plasma volume produced by it are roughly equivalent to the amount administered. In this respect our results might be expected to differ from those reported by Bradley(2) and Cargill(1) where plasma volume expansion was produced by the oncotic action of concentrated albumin solution. A more comparable study is that of Wilson and Harrison(3) who administered 900-1955 cc of plasma to normal human subjects and found that the blood volume was increased in each instance by approximately the amount infused. These authors report changes in exogenous creatinine clearance ranging from —4 to +228 cc/min., with an average of +83 cc/min. C_{PAH} increased in every instance, ranging from +92 to +1008 cc/min. and averaging +514 cc/min. We are unable to explain our failure to find similar changes when the plasma volume is expanded by dextran. It is possible that the renal vasodilatation produced by plasma and albumin is due to the direct action on the kidney of an unidentified substance in these solutions and not to the concomitant increase in plasma volume.

Summary. Following the intravenous administration of 500-1500 cc of 6% dextran solution in 13 normal subjects there were no

marked changes in clearance of PAH and creatinine or in Tm_{PAH}.

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Rapidity of Calcification *in vitro*.* (19461)

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Most experiments reported in the literature on the calcification of hypertrophic rachitic cartilage *in vitro* depend on 16 to 20 hour incubation periods. Generally, these have been performed with minimal concentrations of calcium and inorganic phosphate. We have observed that calcification can proceed with unexpected rapidity in organic phosphate ester, and that the characteristic line of calcification may be observed after as little as 5 minutes incubation.

Methods. Slices of epiphyseal cartilage were prepared from bones removed from young rats, which had been made rachitic by feeding them a modified Steenbock and Black diet No. 2965(1). They were shaken in a water bath at 37°C for varying periods of time in calcification medium, as described by Miller, Waldman, and McLean(2), then stained with 2% $AgNO_3$, and cleared with oil of isosafrol. Phosphate was supplied as inorganic phosphate, from a stock solution of 0.1 M phosphate buffer, pH 7.4, or as disodium monophenyl phosphate. Inorganic phosphate liberated from the organic ester was determined by the method of Gomori(3) after removal of the slices and precipitation of the contents of the flask with an equal volume of 10% CCl_3COOH. The calcification score used is that described by Sobel, Goldfarb, and Kramer(4).

Results. In a medium which contained only inorganic phosphate a wide line of calcification was noted in 5 of 6 slices after 8 hours incubation in the highest concentration of phosphate, 5.0 mM (Exp. 1, Table I). The density of the calcium phosphate deposit increased after 22 hours incubation. Little or no calcification was noted up to this time with 2.0 or 3.0 mM inorganic phosphate. In the presence of organic phosphate ester, however, all the slices showed calcification after only one hour incubation. At this time, the concentration of inorganic phosphate attained in the medium due to hydrolysis of disodium monophenyl phosphate was only 1.7 mM. As in the experiment with inorganic phosphate, the density of the bone salt deposit became greater with more prolonged incubation.

In order to determine how rapidly salt deposit can occur in organic phosphate ester, a second experiment was performed with a higher concentration of Ca^{++}, 2.0 mM. Flasks were removed from the incubator after various intervals of time, from 5 to 60 minutes. Beginning calcification was noted in 1 of 4 slices after only 5 minutes, when the inorganic phosphate content of the medium had reached

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TABLE I. Rate of Calcification of Rat Hypertrophic Rachitic Cartilage Slices in Inorganic and Organic Phosphate.

Exp. No.	Incubation	Ca^{++}, mM	Inorganic phosphate: Conc., mM	Inorganic phosphate: Calcification score	Disodium monophenyl phosphate: Conc., mM	Disodium monophenyl phosphate: Calcification score	Disodium monophenyl phosphate: Inorganic PO_4-liberated, mM
1			2	6=0			
	1 hr	1	3	6=0		3=4+, 1=2(4+), 2=3(4+)	1.7
			5	6=0			
			2	6=0			
	2	1	3	4=0, 2=±		1=3+, 4=2(4+), 1=3(4+)	3
			5	5=0			
			2	5=0			
	3	1	3	7=0	33	1=4+, 4=2(4+)	4.3
			5	5=0, 1=±			
			2	4=0, 1=±			
	8	1	3	4=0, 1=±		3=2(4+), 2=3(4+)	7.2
			5	3=2(4+), 1=3(4+), 1=±			
			2	2=0, 3=±			
	22	1	3	2=0, 2=+, 1=2+		3=2(4+), 2=3(4+)	22.6
			5	2=3+, 1=4+, 2=2(4+)			
2	5 min	2				3=0, 1=3+	.15
	10	2				3=4+, 1=3+, 1=±	.26
	15	2			33	5=2(4+)	.35
	30	2				1=4+, 4=2(4+)	.61
	45	2				2=2(4+), 3=4+	.70
	60	2				1=3+, 2=2(4+), 1=3(4+)	.86

See text for a discussion of the density of calcification.

a level of only 0.15 mM (Exp. 2). The line of calcification was very narrow and extremely faint, but was characteristic and in the normal position. After 10 minutes, 3 of 5 slices showed a narrow line of calcification extending across the provisional zone of calcification (4+), but still extremely faint. After 15 minutes incubation, all the slices exhibited a wide band of calcification which extended across the provisional zone of calcification and included the primary tongue of cartilage. Both the width of the band of calcification and the density of the calcium phosphate deposit increased with time of incubation. After one hour, when calcification was marked, the inorganic phosphate content of the medium had reached a level of only 0.86 mM.

Summary and conclusions. 1. In the presence of organic phosphate ester, calcification of hypertrophic rachitic cartilage slices occurs with great rapidity and at what appears to be an extremely low concentration of inorganic phosphate. The estimated concentration of inorganic phosphate ester is undoubtedly deceptive, because it does not indicate the concentration which prevails in the immediate vicinity of the slice and in the slice itself. This is probably extremely high because of degradation of organic phosphate ester by alkaline phosphatase in the bone slice, and the limited rate of diffusion of phosphate away from the slice. 2. Our results also indicate that in a very short time the outline of the final calcification pattern is formed, and the density of the bone salt deposit then increases with time.

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Metabolic Relationship Between Acetoacetate and Glucose. (19462)

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Glucose has long been known to lessen the extent of ketonuria(1-5). According to Harry *et al.*(6) ketolysis rather than antiketogenesis is apparently the primary mechanism whereby metabolism of carbohydrate brings about diminution of ketonuria. West(7) succeeded in condensing glucose with ethylacetoacetate, *in vitro*, to give rise to a product which was shown to be 2-tetra hydroxy butyl 5-methyl 4-carbethoxy furan (Gonzales (8,9)). Since Shaffer(10) put forward his interesting theory on ketolytic role of glucose, there have been many speculations as to whether glucose or some active derivative of it accelerates combustion or oxidation of the acetoacetate molecule by a definite chemical union of the nature of condensation or by merely exerting a catalytic effect. Soskin and Levine(11) reported that the rate of utilization of glucose is within certain limits proportional to the height of the blood sugar level in normal as well as diabetic animals. It has been found in this laboratory(12,13) that the intermediary fat metabolites when injected in the normal rabbits bring about a rise in blood sugar. As suggested earlier by Nath(14) and recently by Best and Taylor(15), this immediate rise in blood sugar as a result of the acetoacetate injection may be due to a physiological response to combat the disturbed situation and may, therefore, be considered as a protective phenomenon.

In vivo, as well as *in vitro* studies were, therefore, undertaken to throw some light on these points and to explore whether there is any metabolic relationship between glucose and acetoacetate that might exist in the living body as a natural mechanism for the diminution of ketone bodies.

Experimental. In vivo studies. A set of 36 healthy male rabbits, having fasting blood sugar values between 108 and 116 mg per 100 cc of blood, were selected. Blood was taken from the marginal ear veins and blood sugar estimated according to the method of Hagedorn and Jensen(16). Samples were collected after fasting for about 18 hours. All injections were subcutaneous. The Na acetoacetate and glucose injected were always in equimolecular proportions and calculated from the molecular weight of the condensation product of the two. As this compound is not highly soluble in water at 37°C, the aqueous suspension was administered subcutaneously by a bovine needle.

In vitro studies. Glucose 60 mg with 1 cc of whole blood and 20 cc of M/15 phosphate buffer of pH 7.3 was incubated at 37°C for 24 hours. The amount of glucose utilized was then found by estimating the amount of glucose remaining according to the method of Hagedorn and Jensen(16). The above experiment was next repeated with Na acetoacetate and condensation product respectively in different amounts.

Discussion. It is evident from Table I (A and B) that both acetoacetate as well as glucose, when injected in very low doses, cause a distinct rise in blood sugar values. But no such rise could be observed even when a high dose of acetoacetate (341.7 mg/kg) was followed immediately by an equimolecular amount of glucose (496 mg/kg), or, when the condensation product of the two (223.2 mg/kg) was administered subcutaneously, this indicated nicely that there is possibly some metabolic relationship between glucose and acetoacetate in the living body, a finding which is in accord with Hawk(17), that "It appears likely that glucose facilitates acetoacetate combustion in the living organism."

It was also observed that if acetoacetate alone is injected in the high dose, the animals showed signs of uneasiness and restlessness, but when the high dose was immediately followed by its equimolecular amount of glucose, these symptoms soon disappeared. It was further noted that the high dose of acetoacetate brings in the urine of a normal healthy rabbit a considerable increase in specific gravity. But no such harmful effect was ob-

TABLE I. Showing Glucose Tolerance of Animals (Rabbits) on Injection of Acetoacetate and Glucose, Administered in Their Equimolecular Proportions, Individually and One Immediately Followed by the Other, and Also Their Condensation Product.

Dose	No. of animals	Substance inj.	Dose, mg/kg body wt	Avg blood sugar values and stand. dev. (mg/100 cc) after 0 hr	½ hr	1½ hr	2½ hr
Low	5	Na acetoacetate	50	113 ±2	130.4±1.9	140.6±2.4	134 ±2.6
	2	Glucose	72.6	113.5±1.5	126.5±1.5	113.5± .5	113.5±1.5
	5	Na acetoacetate, immediately followed by glucose	50 72.6	115 ±2	106.6±1.9	109 ±2.9	100.6±2.7
	6	Condensation product of glucose and ethylacetoacetate	110.5	113 ±2.4	115 ±1.9	109 ±2.3	101 ±2.8
High	4	Na acetoacetate	341.7	112.8±1.9	175 ±2.7	183 ±3.1	179 ±2.6
	2	Glucose	496	113.5±1.5	169 ±1	102 ±1	113.5±1.5
	6	Na acetoacetate immediately followed by glucose	341.7 496	108 ±3.4	116 ±1.6	111 ±3.5	100 ±4.2
	6	Condensation product of glucose and ethylacetoacetate	223.2	111 ±2.2	115.8±2.4	115 ±2.1	119 ±1.9

TABLE II. Showing Glucose Utilization With and Without Na Acetoacetate or Condensation Product. (60 mg of glucose with 1 cc of whole blood, and 20 cc of M/15 phosphate buffer of pH 7.3 incubated at 37°C for 24 hr with different amount of condensation products and Na acetoacetate respectively).

	No. of experiments	Amt of condensation product (mg)	Amt of Na acetoacetate (mg)	Avg values for glucose utilized (mg) and stand. dev.	Avg % of glucose utilized*
A.	4	0	0	45 ± 2.2	73
B.	2	11.41	0	45 ± 4	73
	2	22.83	0	44 ± 0	72
	2	45.66	0	43 ± 0	71.8
	4	91.33	0	42 ± 3.1	70.6
	3	182.66	0	35 ± 2.9	56
C.	2	0	5.16	45 ± 0	73
	2	0	10.33	42 ± 2	70.6
	2	0	20.66	41 ± 0	70
	5	0	41.33	32 ± 7.2	49.6
	3	0	82.66	21 ± 2.8	29.8
	3	0	123.99	18 ± .255	23.4
D.	4	91.33	41.33	27 ± 5.9	40.6

* In calculating avg % of glucose utilization, blank values obtained in experiments without blood were subtracted from observed values.

served on immediately following the above high dose by its corresponding amount of glucose. Further, when the subcutaneous injections of both substances continued for some days there was no diuresis, but was observed in the animals receiving acetoacetate alone. Also the condensation product of glucose and ethyl acetoacetate administered subcutaneously to a normal rabbit, in the high dose of 223.2 mg/kg, had practically no abnormal effect on the system, *i.e.*, no glucose, acetone, albumin were observed in the urine of treated animals, neither was there diuresis or rise in specific gravity. Further, the high dose of condensation product (300 mg/kg) given subcutaneously to a normal rabbit, fasted for nearly 18 hours, did not raise the ketonemic level from its initial value. The estimation was done by the method of Behre and Benedict(18) at intervals of 2, 4, and 6 hours, respectively.

In vitro studies (Table II) indicated acetoacetate in small amounts of 5.16, 10.33, and 20.66 mg respectively, had practically no inhibitory effect on normal glucose utilization. But in appreciable amount (41.33 mg) it exhibited a distinct inhibitory effect on normal glucose utilization. On gradually increasing

the concentration of acetoacetate, such inhibition became more and more pronounced. On the other hand the condensation product of glucose and ethylacetoacetate was found to be without any inhibitory effect on the normal glucose utilization up to a concentration of 91.33 mg, *i.e.*, an amount equimolecular to 41.33 mg of acetoacetate. With amounts greater than 91.33 mg, the condensation product did show some inhibitory effect but very much less than that exhibited by corresponding amounts of free acetoacetate alone. In one set of experiments (Table II, D) wherein both condensation product and acetoacetate were kept, it was noted that the free acetoacetate together with the chemically combined form (condensation product) shows better utilization of glucose than the corresponding amount of free acetoacetate alone. Thus *in vitro* findings revealed the inhibition of normal glucose utilization by increased concentration of acetoacetate. It appears likely that increased concentration of acetoacetate very probably seriously impairs the metabolic relationship between glucose and acetoacetate, perhaps through the process of inactivation of the enzyme in the system, also obtained by Nath and Hatwalne(19).

Summary. 1. A metabolic relationship between glucose and acetoacetate has been shown to exist in the living body. 2. Increased concentration of acetoacetate (Na salt) distinctly inhibits normal glucose utilization when incubated at 37°C at a pH of 7.3 in the presence of whole blood (rabbit). 3. The condensation product of glucose and ethylacetoacetate has no effect on the normal ketonemic level and blood sugar tolerance curve of a rabbit and has neither any abnormal effect on the urine constituents of the treated animals.

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Growth-Promoting Activity of L-Lyxoflavin for *Lactobacillus lactis*.*† (19463)

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Lyxoflavin was reportedly isolated from human heart muscle by Pallares and Garza (1) and shown to be identical with synthetic L-lyxoflavin. They(2) considered it a "stereoisomer of vit. B_2." Emerson and Folkers(3,4) showed that synthetic L-lyxoflavin(5) possessed a growth-promoting activity distinct from riboflavin activity for rats. Wahlstrom and Johnson(6) reported significantly greater gains in weight of pigs when L-lyxoflavin was incorporated in a diet containing all known vitamins. Bruins *et al.*(7) obtained a 10% weight increase in chicks on a basal diet containing riboflavin, with L-lyxoflavin alone and in combination with dried whey, wheat bran and liver residue. Hendlin and Wall, quoted in Emerson and Folkers(4), found that L-lyxoflavin had less than 0.2% activity of riboflavin for this microorganism. Snell(7) states that lyxoflavin exerted a competitive inhibition against riboflavin for *L. casei* in high concentrations, although it was slightly stimulatory in the presence of suboptimal doses of riboflavin.

Studies reported in this paper show that L-lyxoflavin promotes the growth of *Lactobacillus lactis*.‡ The effect of this compound on the growth of 2 other bacterial species is also discussed.

Experimental. Three cultures have been used in these tests: *Lactobacillus lactis*, ATCC 8000, *Lactobacillus casei* ATCC 7469, both of which require riboflavin under the conditions of assay, and *Leuconostoc mesenteroides* ATCC 8293, which apparently synthesizes riboflavin under the conditions of assay. The riboflavin assays with *L. casei* were carried out by the modified method of Snell and Strong, as described by Snell(8). Turbidimetric measurements were made after 20 to 40 hours incubation using the Evelyn colorimeter fitted with a 620 mμ filter. The stock culture of *Leuc. mesenteroides* was maintained by monthly transfer in Eugon agar stabs (Baltimore Biological Laboratory). The stock culture of *L. lactis* was transferred

TABLE I. Composition of Double Strength Medium.*

	mg		mg
DL-α-Alanine		Adenine sulfate	
L-Arginine mono-HCl		Guanine HCl	2
DL-Aspartic acid		Uracil	
L-Glutamic acid		Xanthine	
Glycine		K_2HPO_4	100
DL-Histidine mono-HCl		KH_2PO_4	100
		$MgSO_4 \cdot 7H_2O$	40
		$MnSO_4 \cdot 4H_2O$	
DL-Isoleucine	40	NaCl	2
L-Leucine		$FeSO_4 \cdot 7H_2O$	
L-Lysine mono-HCl		$CaCl_2$	20
		Thiamin HCl	2
DL-Methionine			
DL-Phenylalanine			μg
		Ca. pantothenate	400
DL-Serine		Vit. B_{12}	.8
DL-Threonine		Pyridoxal phosphate	200
DL-Tryptophane	80		
L-Tyrosine		p-Aminobenzoic acid	400
DL-Valine	40		
L-Asparagine		Nicotinic acid	800
L-Cystine		Biotin	1
Sodium ethyl oxalacetate	100	Pteroylglutamic acid	1
Tween 80	20	KCN	50
		LBF factor†	10 units
	g		
Na acetate, anhyd.	1.2	Distilled water to	100 ml
Dextrose	2		

* Cystine should be dissolved in concentrated HCl and water before adding to dissolved amino acids, kept in stock as a dry mix. Pyridoxal phosphate and sodium ethyl oxalacetate are added after the pH is adjusted to 6.8.

† LBF factor, Lederle 8000 unit concentrate or pantethine (synthetic LBF) Merck.

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‡ *L. lactis* is synonymous with *Bacillus lactis acidi*, according to Bergey's Manual of Determinative Bacteriology, 6th Edition, 1948. Williams & Wilkins Co., Baltimore.

TABLE II. Growth Response of *Lactobacillus lactis, Lactobacillus casei* and *Leuconostoc mesenteroides* to Riboflavin and L-Lyxoflavin.

Substance added	μg/10 ml	*Lactobacillus lactis*		*Lactobacillus casei*		*Leuconostoc mesenteroides*	
		Incubation period, hr					
		22	40	20	40	22	40
		Galvanometer reading*					
0	.0	92	91	94	94	77	44
Riboflavin	.025	76	69				
	.05	67	57	78	81	71	41
	.1	58	41	66	67	65	42
	.25			49	47		
	.3	53	36				
	.5	50	35	47	38	60	40
L-Lyxoflavin	.05	91	87			93	95
	.1	83	73	92	96	94	97
	.3	65	45				
	.6	55	39			95	98
	1	54	36	92	93	96	96
	5	61	37	91	94	96	97
	10			94	94	96	89
	50	62	32	94	94	96	69
	100	62	37				

* Galvanometer reading of 100 represents no growth.

biweekly on a stock medium consisting of 10% clarified tomato juice, 0.5% Bacto-yeast extract, and 10% Bacto-dehydrated skim milk (adjusted to pH 6.8 and sterilized at 121°C for 15 minutes). Both organisms were grown in 10 ml of Difco Micro Inoculum broth for the inoculum. After 18 to 24 hours incubation at 37°C, the cultures were washed twice with physiological saline, resuspended in saline and adjusted to a turbidity reading of 70 on the Evelyn colorimeter (515 mμ filter). Four drops of this suspension were added to 20 ml of saline for the final inoculum, and one drop was used per tube. The composition of the basal medium used with *L. lactis* and *Leuc. mesenteroides* is shown in Table I. This medium, supplemented with 20 μg of riboflavin, was developed in a study of an unidentified growth stimulating factor for *L. lactis*(9). Complete growth of *L. lactis* is not reached until about 40 hours incubation if this factor is not supplied. Since no pure source of the stimulatory factor is available, a 40-hour incubation period was used in most of the *L. lactis* assays to permit the organism to synthesize the factor. The final volume was 10 ml, 5 ml of double strength medium being added to 5 ml of sample. The tubes were autoclaved for 5 minutes at 121°C, cooled, inoculated and incubated at 37°C for varying periods of time, depending on the assay. Growth was determined turbidimetrically with the Evelyn colorimeter, using the 620 mμ filter.

Results and discussion. The growth responses of *L. lactis, L. casei* and *Leuc. mesenteroides* to riboflavin and L-lyxoflavin are shown in Table II. While L-lyxoflavin promotes the growth of *L. lactis,* the rate of growth is somewhat slower than that with riboflavin. The flattened growth curve at 22 hours was obtained because, in this medium, the stimulatory factor must be synthesized by the organism. There was slight inhibition with 5 to 100 μg per tube of L-lyxoflavin at 22 hours, but no inhibition was evident at 40 hours with these doses. When the incubation period was continued to 3 or 6 days, the activity of L-lyxoflavin approached that of riboflavin. In a series of eight assays with *L. lactis,* L-lyxoflavin had an average activity per μg equivalent to 0.09 μg of riboflavin in a 20- to 24-hour assay; 0.16 μg at 40 to 44 hours and 0.43 μg in 6 days.

While *L. casei* gave a typical dosage response curve to riboflavin after 20 hours of incubation, no growth was obtained with *L-lyxoflavin* even after 72 hours' incubation.

TABLE III. Inhibition of Growth of *Lactobacillus casei*, *Leuconostoc mesenteroides* and *Lactobacillus lactis* by L-Lyxoflavin in Presence of Riboflavin, after 40 Hr Incubation.

Riboflavin added, μg/10 ml	L-lyxoflavin added, μg/10 ml	*Lactobacillus casei* Snell's medium GR*	II†	*Lactobacillus casei* Synthetic medium GR*	II†	*Leuconostoc mesenteroides* Synthetic medium GR*	II†	*Lactobacillus lactis* Synthetic medium GR*	II†
0	0	98		86		44		93	
0	.05	-		80		95		93	
0	50	97		75		69		41	
.05	0	82		80		41		67	
.1	0	72		58		43		48	
.5	0	64		52		40		42	
1	0	64		55		47		41	
.05	.05					86	.37	57	
.05	.1	76	60			92		53	
.05	.5	93				92		40	
.05	50	97				96		39	0
.1	.1	65		55		77	.33	43	
.1	1	63		52		84		39	
.1	5	78	50	55		85		38	
.1	10	93		58		85		39	
.1	50	97		92	440	93		40	0
.5	.5	61		50		39		39	
.5	1	55		50		42		40	
.5	10	57		50		48		40	
.5	50	92	50	57	>100	65	150	39	0
1	1	67		53		48		41	
1	5	60		52		50		41	
1	10	57		50		50	–	41	
1	50	75	50	48	0	47	0	41	0

* GR—A galvanometer reading of 100 represents no growth.

† II—Inhibition index is equal to the ratio of concentration of inhibitor to concentration of the metabolite, at which half maximum inhibition of growth of the organism occurs. The inhibition index was determined on curves drawn from the data shown in the table.

Riboflavin was not required by *Leuc. mesenteroides* at 22 hours, but at this time it had a slight stimulatory effect at low levels. L-lyxoflavin was very active in inhibiting the growth of *Leuc. mesenteroides* at this early hour, over a range of from 0.05 to 50 μg per tube. However, at 40 hours, riboflavin was still only slightly stimulatory while *L. lyxoflavin* at the higher levels produced a response which must be interpreted as growth stimulation in the light of inhibition analyses which follow.

Inhibition analyses were made with all 3 organisms and results are shown in Table III. In confirmation of Snell's report(7), *L. casei* was competitively inhibited by L-lyxoflavin when Snell's assay medium for riboflavin was used, and slight stimulation occurred at the lower levels of lyxoflavin in the presence of suboptimum riboflavin. However, when the same tests were done with *L. casei,* using the synthetic medium described in Table I, the inhibition with L-lyxoflavin was evident only when low levels of riboflavin were in the medium. When high levels of riboflavin were used, high levels of L-lyxoflavin stimulated the growth of *L. casei.* More marked growth stimulation occurred with 50 to 100 μg per tube of L-lyxoflavin in the synthetic medium containing 1 μg per tube of riboflavin when the *L. casei* inoculum was washed and diluted by the method used for *L. lactis* (data not shown). The response with a washed dilute culture suggests that adaptation occurred or that some component in the synthetic medium, in addition to riboflavin, might be required for L-lyxoflavin utilization. Since the treatment of the components of Snell's medium would tend to destroy or remove some of the vitamins present in the synthetic medium, it is possible that one of these vitamins is involved.

With *Leuc. mesenteroides,* inhibition was competitive only at the lowest levels of L-

lyxoflavin used. At high levels of L-lyxoflavin the inhibition disappeared. There was no evidence of competitive inhibition with *L. lactis*.

Among other pentose isoalloxazines or probable isoalloxazine precursors examined for growth stimulation or inhibition for *L. lactis* and *L. casei*, D-lyxoflavin, D-araboflavin(10), galactoflavin and α-ribazole did not promote growth of either organism, while 5,6-dimethylbenzimidazole was inactive for *L. lactis* (not tested with *L. casei*). Only riboflavin phosphate(11) and isoriboflavin(12) had activity, having 0.82 and 0.038 μg of riboflavin activity per μg; respectively, for *L. casei*, and 0.66 and 0.002 μg activity for *L. lactis*. The former compound was not corrected for phosphate content. All of the above compounds were tested for inhibition, using 1 μg of riboflavin in the medium per tube, in Snell's medium with *L. casei*, and in the synthetic medium with *L. lactis*. No inhibition was observed with any of the compounds, nor was there any stimulation over that due to the added riboflavin.

A microbiological assay for the presence of L-lyxoflavin in natural materials does not appear at present feasible when using either growth stimulation or competitive inhibition as a criterion. Because all of the pentose isoalloxazine compounds migrated at the same rate in several solvent systems, paper chromatographic separation of L-lyxoflavin from the other pentose isoalloxazines has not been accomplished, although this would have been of great value in a microbiological assay. The method of synthesis of L-lyxoflavin makes it highly improbable that it is contaminated with riboflavin. Therefore the growth of *L. lactis* with L-lyxoflavin must be due to a direct response to this compound. Furthermore, in other tests, it has been observed that the total riboflavin content of an *L. lactis* culture was not increased when it was grown in the presence of lyxoflavin. The fact that *L. casei* responds to L-lyxoflavin, when riboflavin is present and the medium is adequate in other nutrients, lends support to the idea that L-lyxoflavin may function in a manner different from riboflavin. The growth response in animals, which has also been observed in chicks in this laboratory,§ may be due to the utilization of L-lyxoflavin directly by the animal body or by micro-organisms. Furthermore, any inhibitory effect of L-lyxoflavin on bacteria might possibly be reversed by the presence of adequate riboflavin and other nutrients in an enriched environment such as would be found in the intestinal tract.

Summary. L-lyxoflavin had 16% of the activity of riboflavin in promoting growth of *Lactobacillus lactis* in a 40-hour assay. The activity increased to 43% after 6 days of incubation. Growth with L-lyxoflavin occurred in the presence or absence of riboflavin and there was no evidence of competitive inhibition. L-lyxoflavin inhibited riboflavin competitively in the growth of *Lactobacillus casei*, in Snell's riboflavin assay medium, with an inhibition index of 50, but the inhibition became stimulation in a synthetic medium when optimum amounts of riboflavin were present. D-lyxoflavin, D-araboflavin, galactoflavin, and α-ribazole were inactive in promoting growth of either *L. lactis* or *L. casei* while 5,6-dimethylbenzimidazole was inactive for *L. lactis*. Riboflavin phosphate and isoriboflavin had riboflavin activity for both organisms.

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Effect of Semliki Forest Virus on Rabbit Myxoma.* (19464)

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Although there is much evidence that indicates that rabbit fibroma virus is closely related to rabbit myxoma virus, the end results of infection caused by these viruses are diametrically opposite(1). Myxoma virus causes a disseminated disease that is usually 100% fatal, while fibroma virus causes localized, benign lesions that regress in 20 to 30 days(2). The destructive effect of Semliki Forest virus (SFV) on rabbit fibromas has recently been described(3). Virus-induced fibromas of domestic rabbits became necrotic and regressed rapidly after the intramuscular injection of SFV. Subsequently, the effect of SFV on rabbit myxoma was studied. This paper describes the results of these studies and the multiplication of SFV in tissue cultures of myxoma subcutaneous tissue.

Methods. SFV was prepared as 20% suspensions of infected mouse brain. The intracerebral LD_{50} titer in 15 g mice varied from $10^{-7.5}$ to $10^{-9.5}$. The myxoma virus, originally isolated by Dr. A. Moses, was obtained from Dr. M. H. D. Smith. In these experiments, this strain of myxoma virus killed all 200 control rabbits that were infected with 10 to 10000000 LD_{50} virus. Death usually occurred 10 to 12 days after infection. Myxoma infection was usually induced by injecting 0.1 ml of a $10^{-4.0}$ to $10^{-5.0}$ dilution of virus (containing 10 to 100 LD_{50}) intradermally on the abdomen of rabbits. New Zealand white rabbits were used for all experiments. The effect of SFV on myxoma was studied by injecting intramuscularly 1.25 ml of a 20% extract of mouse brain SFV or a 20% extract of myxoma-passed SFV (that also contained myxoma virus) 24 hours after infecting the rabbits with myxoma. Rabbits were housed in an air-conditioned room maintained at 20°C. The development of myxoma lesions was observed daily. Rabbits were discarded and considered recovered from myxoma infection when all the lesions had disappeared 21 to 55 days after the initiation of infection. Weighed portions of myxoma lesions were extracted, centrifuged and titrated intradermally on the abdomens of normal rabbits using 3 to 4 inoculations per dilution and a 0.1 ml inoculation. Weighed blood clot was titrated in a similar manner when quantitative estimations were made of myxoma viremia. SFV rabbit antiserum was added to extracts of myxoma tissues that contained SFV before myxoma titrations were performed. Myxoma virus dilution neutralization tests were performed by incubating equal amounts of serum and virus one hour at 24°C, and titrating intradermally on the abdomens of rabbits. The tissue culture technic has been previously described(3).

Results. Multiplication of SFV in tissue culture. The multiplication of SFV in minced myxoma subcutaneous tissue resembled that of SFV in fibroma tissue. After the inoculation of 10 to 400 LD_{50} SFV, maximum titers of $10^{-5.5}$ to $10^{-6.5}$ developed in 48 to 96 hours and persisted at least 8 days. However, little or no multiplication of SFV was demonstrated in 15% of the experiments. Detailed studies were undertaken to investigate these inconsistencies in the growth of SFV. SFV multiplied equally well in 0.2 and 0.4 g amounts of 4 to 9 day subcutaneous myxoma tissue. The yield of SFV was not affected by the passage of myxoma virus used to initiate the myxoma tissue. Myxoma-passed SFV did not multiply better than mouse brain SFV. Both the white, homogenous, upper part and the lower, gelatinous part of the myxoma growth supported equally well the multiplication of SFV. The actual cause for irregular SFV multiplication was never completey revealed. However, best growth was observed when the inoculum of SFV was of the order of 200-400 LD_{50} and when myxoma tissue was minced into 1 x 2

* This investigation was supported by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

ml rather than 0.05 to 1 ml pieces.

Effect of mouse brain SFV on course of myxoma. In preliminary experiments, SFV from infected mouse brain was injected intramuscularly into rabbits 1 to 3 days after the intradermal inoculation of 1000 to 100000 LD_{50} myxoma virus. In some of the SFV-injected rabbits, certain observations suggested that myxoma was modified by SFV even though all the rabbits died with signs of generalized myxoma infection. On occasion, death was prolonged 4 to 8 days beyond the controls' usual survival period. The initial lesions that developed at the site of myxoma virus inoculation became smaller and occasionally necrotic, and the ear lesions were sometimes circumscribed. When mouse brain SFV was injected intramuscularly one day after intracutaneous injection of 10 to 100 LD_{50} myxoma virus, 5 to 10% of the rabbits survived after undergoing a generalized myxoma infection. Delayed death, initial lesion involution, and circumscribed ear lesions were observed more commonly when 10 to 100 LD_{50} myxoma inocula were employed. The effect of SFV on myxoma was more clearly demonstrated in normal rabbits by a combined intramuscular injection of 1.25 ml of 20% mouse brain SFV and 20% myxoma (Table I). Eight of 21 injected rabbits recovered completely after developing generalized myxoma.

Effect of myxoma-passed SFV on course of myxoma. SFV was passed 45 times in myxomatous rabbits to determine whether adaptation of SFV to myxoma would enhance the effect of SFV on myxoma. 1.25 ml amounts of 20% SFV extract were injected intramuscularly into rabbits bearing 3- to 5-day-old abdominal growths. The abdominal myxoma lesions, harvested 2 days after the inoculation of SFV, were used as the source of the SFV for the next passage. Various passages were injected intramuscularly into normal rabbits and into rabbits infected 24 hours earlier with 10 to 100 LD_{50} myxoma virus. The results of the tests are depicted in Tables I and II. All tested extracts of myxoma-passed SFV caused a further significant reduction in the myxoma fatality rate when injected intramuscularly with myxoma into normal rabbits.

TABLE I. Result of Combined Intramuscular Injection of SFV and Myxoma Virus in Normal Rabbits.*

Inoculum	D/I†	Myxoma fatality rate, %
Control—Myxoma virus	15/15	100
Mouse brain SFV and myxoma	13/21	62
10th passage SFV in myxoma and myxoma	1/8	12
21st	4/18	22
31st	5/14	36
43rd	6/18	33
45th	1/10	10

* 1.25 ml of 20% SFV and 20% myxoma.

† Numerator=rabbits that died; denominator=rabbits inoculated.

TABLE II. Effect of SFV on Rabbits Infected with 10 to 100 LD_{50} Myxoma Virus.*

Inoculum	D/I†
Control—Myxoma virus	70/70
Myxoma passed SFV—3rd passage	2/4
10th	5/6
43rd	26/29
45th	18/35

* 1.25 ml of 20% SFV extracts were inj. intramusc. 24 hr after intracutaneous infection with myxoma virus. Extracts of myxoma passed SFV also contained myxoma virus.

† Numerator=rabbits that died; denominator=rabbits inoculated.

However, in rabbits previously infected with myxoma, only passage No. 45 substantially decreased the myxoma fatality rate. Subsequent tests confirmed this special effect of passage No. 45 SFV extract. No evidence was obtained that myxoma had become attenuated during the repeated passages of SFV. Injection of 0.1 ml of the 45th passage of SFV in myxoma, containing 10 LD_{50} myxoma virus and sufficient SFV antiserum to neutralize completely the SFV present, killed 19 of 19 rabbits in the usual 10- to 12-day period. During the passages in myxoma, the titer of SFV ranged from $10^{-7.5}$ to $10^{-9.5}$. SFV was separated satisfactorily from myxoma of passage No. 45 SFV-myxoma only by inoculating the virus mixture intraperitoneally in mice. When the brains were harvested, SFV was present in a titer of $10^{6.5}$ LD_{50}, but no myxoma virus was demonstrable. Intramuscular injection of this 20% SFV mouse brain extract did not prevent death in

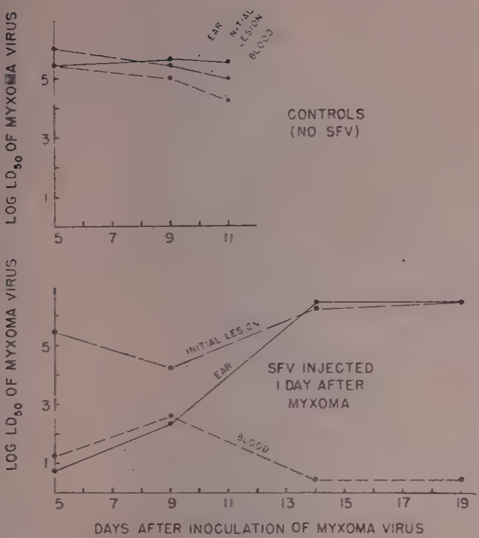

FIG. 1. Effect of SFV on recovery of myxoma virus. Each point represents average of myxoma virus concentrations in 3 or 4 rabbits. Determinations were terminated in control animals on the 11th day when the rabbits were dead or moribund.

17 of 17 domestic rabbits inoculated one day earlier with 50 LD_{50} myxoma virus.

Recovery of virus from myxomatous rabbits. Abdominal lesions, sections of ear, and blood were removed at intervals from a group of myxomatous rabbits inoculated with the 45th passage of SFV in myxoma and from a control group of myxomatous animals injected with 20% myxoma virus intramuscularly. Titrations were made for SFV and myxoma virus. SFV persisted 13 to 18 days in the abdominal lesions at titers of $10^{3.0}$ to $10^{4.5}$ LD_{50}, whereas no SFV was detected in blood or ear 4 days or later after the inoculation of SFV. Fig. 1 illustrates the findings with regard to myxoma virus.

Mechanism of action of SFV on myxoma. Intramuscular injections of heat inactivated (65°C for one hour) SFV and normal mouse brain had no effect on the course of myxoma. Intramuscular injection of myxoma virus did not interfere with the development of generalized myxomatosis subsequent to the intracutaneous inoculation of myxoma. Because SFV did not significantly modify the initial body temperature response to myxoma infection, there is no evidence that elevated body temperature *per se* was responsible for the modification of myxoma(4). Earlier and more marked necrosis was seen in histologic sections of myxomatous lesions of SFV-myxoma inoculated rabbits than in tissues from control myxoma rabbits. Low titers of myxoma neutralizing antibody (neutralization of 1 to 2 log LD_{50} of myxoma virus) was detected 14 to 19 days after onset of infection.

Neurologic sequella of SFV injection. Thirty per cent of all rabbits injected intramuscularly with mouse brain or myxoma-passed SFV developed neurologic sequella, usually paralysis of one or more limbs. Ten per cent of all SFV-injected rabbits died from neurologic complications 5 to 6 days after injection of SFV. These deaths were easily distinguished from myxoma deaths and were excluded in determining myxoma fatality rates.

Discussion. The reported experiments demonstrate that intramuscular injection of SFV can reverse the usually 100% fatality rate of myxoma virus infections. Although this effect was demonstrated to some extent with SFV from infected mouse brain, it was best effected with myxoma-passed SFV. Efforts to study the mechanism of the superior myxoma destroying effect of myxoma-passed SFV were thwarted because SFV could not be separated from the myxoma without further modification of the SFV. SFV probably exerted its effect on myxoma infection by causing necrosis of myxoma tissue and, consequently, reducing myxoma viremia and metastatic myxoma lesions.

Summary. Semliki Forest virus multiplied in suspended cell cultures of myxoma tissue to titers of $10^{5.5}$ to $10^{6.5}$ LD_{50} in 48 to 96 hours. In contrast to the usual 100% mortality in myxoma infection, intramuscular inoculations of mouse brain SFV combined with myxoma reduced the myxoma fatality rate to 62%. Myxoma-passed SFV and myxoma injected together intramuscularly killed only 25% of the rabbits. In rabbits that had been infected intracutaneously with myxoma virus 24 hours earlier, intramuscular injection of the 45th passage of SFV in myxoma reduced the

myxoma fatality rate from 100% to 51%.

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Effect of Polyvinylpyrrolidone on Reticulo-Endothelial Storage. (19465)

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Polyvinylpyrrolidone (PVP), a macromolecular substance first synthesized in Germany during World War II, has been used in Europe as a plasma substitute, reportedly with good results. Abstracts of the extensive European literature on PVP have been presented in a monograph(1) issued by the General Aniline and Film Corporation. Recently the possible use of PVP as "plasma expander" has been considered in this country. Hence biologic properties of PVP deserve continued investigation. Reports on storage of PVP in tissues are contradictory. Earlier negative findings were obtained on dogs and man (Korth and Heinlein(2)). On the other hand, Bargmann(3) observed storage in splenic macrophages of dogs and rats, and Barfuss and Eichler(4) noted transitory storage in rabbits, guinea pigs, and rats. Ammon and Müller(5) found evidence of PVP storage in rabbits, but not in infants treated with PVP, whereas Schoen(6) reported positive findings in such infants. Equivocal results were reported by Riedel and Zipf(7), and by Pellerat *et al.* (cit. 1, p. 50). Recently Nelson and Lusky(8) found in rabbits injected with PVP slight splenic enlargement, and presence of foam cells in spleen, lymph nodes, bone marrow, and other tissues. Bennhold, Schubert, and associates(9-12) reported an interesting property of PVP, namely its ability to promote renal excretion of dyes that are otherwise not excreted at all, or are excreted through the bile. Schubert(12) also found that PVP was capable of "washing out" dyes from tissues with subsequent renal excretion of the dye.

The present investigation is concerned with the effect of PVP on reticuloendothelial storage of dyes in mice. Since previous work (13-15) had shown that the extent of reticuloendothelial storage in mice may vary according to the strain, animals of several inbred strains were utilized for this study.

Materials and methods. Male and female mice, 3 to 6 months old, of strains C57 Black, dba, C3H, and CBA were used. The animals were free of tumors and any other obvious disease. They were kept on *ad libitum* diet of Rockland mouse pellets and water. The dyes used were 1) Erie Fast Rubin Conc. (EFR),* described in a previous communication(15), and 2) Chlorazol Black E (CBE). As a rule, 0.5 ml of 0.5% solutions of dye was injected subcutaneously 2 to 3 times weekly. This dosage was well tolerated by the animals, except in some instances where it was necessary to diminish the quantity of EFR. Controls and experimental animals received equal amounts of dye. PVP† was used as 3.5% solution in physiologic saline, of which 0.5 ml was injected daily subcutaneously, or intraperitoneally in some of the earlier experiments. The animals were weighed at least once weekly, and at the end of the experiments. They were sacrificed by decapitation, permit-

* This dye was obtained through cooperation of the National Aniline Division, Allied Chemical and Dye Corp.

† Mr. D. Witwer, General Aniline & Film Corp., furnished a generous supply of PVP. All experiments were carried out with a single lot (GA-PVP-118).

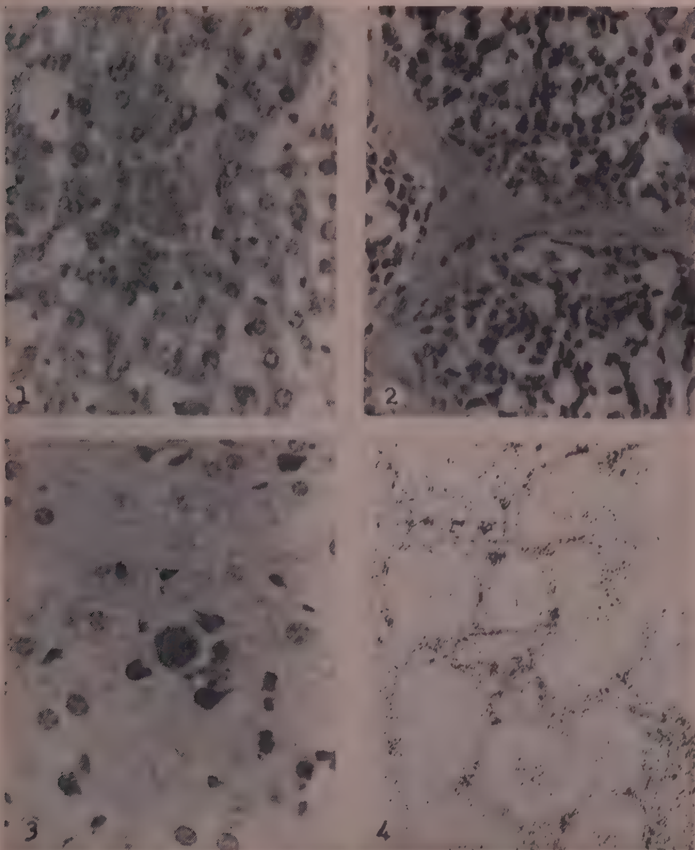

FIG. 1. Liver, C3H male, 18 injections of PVP. Note vacuolation and distention of Kupffer cells. H & E. × 300.

FIG. 2. Spleen. Same animal as Fig. 1. Extensive foam cell accumulation. H & E. × 300.

FIG. 3. Liver, C57 Black male (C-88). Control injected with EFR. Coarse granular dye storage in Kupffer cells. H & E. × 300.

FIG. 4. Spleen, C57 Black male (C-109). Control injected with CBE. Heavy perifollicular dye storage. Unstained. × 30.

ting collection of blood. Autopsies were done, the spleen weighed, and liver, spleen, kidney, lung, and skin fixed in formalin. Sections of these organs were stained with hematoxylin and eosin, and in experiments involving administration of dyes, also unstained sections were prepared in order to permit closer study of dye deposits. In some experiments the method of Chalkley(16) was used for quantitative estimation of dye storage. Details of this technic will be given later. During and after periods of injection of the dye the urine was watched for its appearance daily, and after sacrifice of the animals the coloration of the serum was recorded.

Results. In preliminary experiments, from 12 to 24 daily intraperitoneal or subcutaneous injections of 3.5% PVP were given to 45 C3H, 40 C57 Black, and 20 dba mice of both sexes. The animals showed no untoward effects from this treatment and continued to gain weight. They were sacrificed within from one to 3 days after the last injection of PVP. Moderate enlargement of the spleen was found, with the enlargement most pronounced in strain C3H and least in C57 Black. Tissue sections revealed considerable vacuolation and "ballooning" of Kupffer cells

in the liver (Fig. 1), and formation of similar vacuolated macrophages in the perifollicular areas of the spleen (Fig. 2), and in the reticulum of lymph nodes. The changes increased with dosage of PVP. They were most marked in C3H mice, while C57 Black animals presented them only after a minimum of 18 injections of PVP. The substance responsible for the appearance of the foam cells is presumably PVP or a derivative of it. Direct histologic identification of PVP is rendered difficult by its ready solubility in water and alcohol, which cause it to disappear from fixed and sectioned tissue. In accord with findings of Nelson and Lusky(8) imprints of fresh tissue stained with Gram's iodine showed a deep brown color of cells presumably containing PVP. Attempts to find such iodophilic material in tissue sections were unsuccessful in our hands.

Having thus found presumptive evidence for storage of PVP in reticulo-endothelial tissues of the mouse, the effect of PVP on storage of dyes was tested in three experimental arrangements: (a) simultaneous administration of dye and PVP; (b) administration of PVP after dye injection; (c) administration of PVP prior to injection of dye.

A. Experimental data and gross findings resulting from *simultaneous administration of PVP and EFR* are given in Table I. Histologic findings in the control group were identical with those made in the course of earlier studies(15). There was heavy storage of dye, mostly in form of coarse granules, in the Kupffer cells of the liver (Fig. 3), in perifollicular macrophages and littoral cells of the spleen (Fig. 4), and variable degrees of histiocytic storage in skin and lymph nodes.

TABLE I. Simultaneous Administration of PVP and Dye (.5 ml of .5% EFR).

	Strain C57 Black			
	Exp. C-69		Exp. C-78	
	Control group	Exp. group	Control group	Exp. group
No. of animals	5	5	7	7
Sex	♀	♀	♂	♂
Solvent of dye	Saline	PVP	Saline	PVP
Route of inj.	i.p.	i.p.	i.p.	i.p.
No. of inj.	7	7	5	5
Color of urine	Yellow	Red	Yellow	Red
" " serum	"	Purple	"	Purple

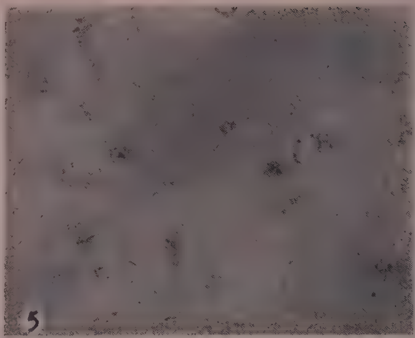

FIG. 5. Liver, C57 Black male (C-78). Exp. animal injected with EFR dissolved in PVP. Note absence of granular storage, and presence of "dye-vacuoles." Unstained. × 250.

The dye deposits were readily visible in hematoxylin-eosin stained slides. In the kidney only occasionally a faint staining of collecting tubules was noted; this was visible only in unstained preparations. In contrast, animals injected with dye dissolved in PVP showed hardly any dye deposits in hematoxylin-eosin stained sections of liver and spleen, while unstained sections revealed macrophages containing a pale red rim of dye around vacuoles apparently produced by intake of PVP (Fig. 5). On the other hand, the renal cortex presented grossly a reddish stain which on microscopic examination was found to be due to dye deposits in the tubular epithelial lining. This is obviously a corollary of the dye excretion noted in the urine of the PVP injected animals.

B. Experimental procedures and gross findings pertaining to the *administration of PVP after the injection of dyes* are summarized in Table II. In experiments C-88 and C-97 control animals were divided into 2 groups; one receiving no treatment after the initial dye injections, the other receiving subcutaneous injections of 0.5 ml of physiologic saline parallel with injections of the same quantity of PVP given to the experimental group. Since animals of the 2 groups showed identical findings, they were combined as one group, and later experiments did not include saline-injected controls. The gross findings in this type of experiment were quite similar to those obtained when dye and PVP were given simultaneously. This applied also when

TABLE II. Administration of PVP after Dye Storage.

No.	Strain	Sex	Group	No. of animals	Dye and dosage (ml)	No. of PVP inj.	Color of serum	Dye storage
C-88	C57 Black	♂	C*	11	EFR 9× .5	0	Y†	3+
			Exp.	6		12	P‡	1+
97	C3H	♂	C	11	" 9× .5	0	Y	2+
			Exp.	6		12	P	1+
110	C57 Black	♂ & ♀	C	6	" 3× .5	0	Y	3+
			Exp.	7	+3× .3	18	Deep P	1+
116	CBA	♂	C	9	" 5× .5	0	Y to pink	2+
			Exp.	9	+4× .25	15	Deep P	±
118	"	♂	C	10	CBE 3× .1	0	Y	1+
			Exp.	10		15	Y	±

* C = control. † Y = yellow. ‡ P = purple.

TABLE III. Effect of PVP on Dye Storage.

No.	Strain	Group	No. of animals	Total storage Range	Mean	± S.D.	Coarse granules Range	Mean	± S.D.
C-88	C57 Black	EFR	11	22–40	32.6*	4.3	16–31	22.5†	4.1
		" +PVP	6	19–34	27.3	6.1	0–8	4 †	2.6
97	C3H	"	11	19–34	25.3*	3.7	15–24	18.5‡	2.7
		" +PVP	6	14–26	21.3	4.7	1–8	4 ‡	2.5

*, †, ‡ Difference between paired values statistically significant.

Chlorazol Black E was used (C-118). The cellular distribution of this dye was similar to that of EFR. Evidence for a displacement of dye from its previous sites of storage was furnished by the appearance of dye in serum and urine. Histologic examination of animals injected subsequently with PVP disclosed a marked decrease in reticulo-endothelial dye storage. The diminished dye content of tissues was due to a change in the type of storage rather than to a decrease in the number of storing cells: instead of the dark red coarse granules in controls, PVP-treated mice presented predominantly either fine granules of dye, or "dye-vacuoles," similar to those noted after *simultaneous* administration of dye and PVP.

In experiments C-88 and C-97, the extent and type of dye storage was estimated by means of the Chalkley tissue analysis(16). Hematoxylin-eosin stained tissue sections of uniform thickness (6 μ) were examined under oil immersion. The reference pointer was moved at random and placed on nuclei of liver cells; a total of 200 nuclei were aimed at in each section. Hits of the 4 pointers on dye material were recorded in 2 categories: (a) coarse granules, (b) other forms of storage (fine granules or dye-vacuoles). Table III shows that administration of PVP brought about a highly significant depression of coarse dye storage. Incidentally, a difference in dye storage, found on statistical analysis to be significant, was also found between control animals of strains C57 Black and C3H, with the former exceeding the latter in storing ability. This corroborates earlier observations on greater storage of carmine in C57 Black than in C3H mice(13,14).

C. Table IV lists data on mice injected with *dye (CBE) after PVP administration.* The reticulo-endothelial storage of CBE, found in untreated mice of all 3 strains, was uniformly prevented by preceding PVP administration (18 injections). Evidence of PVP storage in the form of "foam cells" was prominent in liver and spleen, being most pronounced in mice of strain C3H, and least marked in C57 Black animals.

Discussion. These experiments indicate that

TABLE IV. Administration of Dye after PVP (1 ml of 1% CBE).

Strain	Group	No. and sex of animal	Color of— Urine	Color of— Serum	Dye storage
C57 Black	C*	5 ♂	Y†	Y	2+
	PVP	10 ♂	Light P‡	Smoky G§	—
C3H	C	5 ♂	Y	Y	+
	PVP	10 ♂	Light P	Smoky G	—
dba	C	5 ♂	Y	Y	+
	PVP	10 ♂	Light P	Yellow to G	—

* C = control. † Y = yellow. ‡ P = purple. § G = gray.

TABLE V. Comparative Dosage of PVP.

Species	Approx. wt, kg	3.5% PVP, ml	PVP, g/kg	No. of inj.
Man	70	1000	.5	?
Rabbit*	3	30	.35	16/8 wk
Mouse	.025	.5	.7	12-24/2-4 wk

* Nelson and Lusky(8).

PVP, or a derivative of it, is stored in reticulo-endothelial tissues of the mouse, leading to formation of vacuolated and distended cells. The extent of storage appears to vary with the strain, inasmuch as C57 Black mice exhibited less PVP storage, or exhibited it only after doses of PVP larger than those required for C3H or CBA mice. In previous work(13,14) C57 Black mice showed a superior storing ability for dyes as compared with C3H mice, and in studies of Davidsohn and Stern(17-20) they exceeded most other strains in production of hemoantibodies.

In attempts to evaluate the biologic activity of PVP, a comparison of dosage in animal experimentation and in clinical use should be included. According to Table V, the g/kg amount of PVP for single administrations is similar for the 3 examples listed. However, the total *amount of PVP* given will obviously depend on the *frequency* of administration.

The results of simultaneous administration of dye and PVP can be interpreted as indicating renal excretion of a portion of the dye, *before* it had a chance to be deposited in reticulo-endothelial tissues, while the remainder of the dye was stored together with PVP. The observations in mice injected with PVP *after* administration of dye appear to indicate, in accord with similar findings by Schubert (12), that dye already ingested by macrophages can be dislodged by subsequently administered PVP. Thus eluted dye reappears in the circulation, and is at least partially excreted in urine. It is possible that some of the circulating dye may be again taken up by reticulo-endothelial cells. This may explain the presence of "dye-vacuoles," representing a mixture of dye and PVP, which was found in this type of experiment as well as after simultaneous administration of dye and PVP. Finally, a "blocking" effect on the storing ability of reticulo-endothelial tissue was observed in mice injected with PVP *prior* to administration of dye. The latter finding is paralleled by observations of Barfuss and Eichler(4) who reported an impaired reticulo-endothelial activity in rabbits injected with PVP, as expressed in delayed disappearance of Congo Red from the circulation.

These experimental findings suggest the following tentative generalizations regarding biologic properties of PVP: 1) it enhances renal excretion of dyes; 2) it can displace and elute at least partially certain dyes from their site of storage; 3) preceding storage of PVP may prevent dyes from being deposited in reticulo-endothelial tissues. If it is permissible to consider the dyes used in our experiments as models of macromolecular substances in general, a number of possible implications can be considered. Schubert(12) proposed to utilize PVP for promoting rapid elimination of toxins (diphtheria, botulinum), and quoted several reports in which this procedure was used with favorable results in animals as well as clinically. The "eluting" effect of PVP on colloids previously stored in reticulo-endothelial tissue is in need of additional investigation. It is possible that the displacement by PVP of stored material depends on chemical and/or physical properties of the latter. Experiments with hemosiderin, thorothrast, India ink, and a number of additional dyes are under way to test this assumption. The inhibition of dye storage by PVP raises the question whether this signifies an impaired functional activity of reticulo-endothelial tissue. Preliminary results of immunization experiments suggest that mice of some strains, after treatment with PVP produced lower

levels of hemoantibodies than controls; these findings, however, are not yet conclusive.

All experiments described in this report were terminated shortly after the last PVP administration. For this reason no statement can be made as to the duration of the storage of PVP. It still remains to be established whether morphologic and functional changes attributable to the administration of PVP are wholly or partially reversible, or permanent.

Summary. 1. Storage of PVP, or of a derivative of PVP, was observed in mice after 12-24 injections of a 3.5% solution. The extent of storage appeared to depend on the mouse strain. 2. Simultaneous administration of PVP and sulfonated azo dyes led to renal excretion of dye, and to a decrease in reticulo-endothelial dye storage. 3. PVP administered after dye was given resulted in elution of dye from reticulo-endothelial tissues. 4. PVP administered prior to dye injection prevented storage of dye. 5. On the basis of these findings, an attempt was made to evaluate biologic properties of PVP.

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Studies on Platelets. IV. A Thrombocytopenic Factor in Normal Human Blood, Plasma, or Serum.* (19466)

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In abnormal conditions, individuals may develop sensitivity to normal plasma and respond to its administration with a series of symptoms which have been grouped under the definition of "plasma transfusion reaction" (1). This has been found to occur consistently in paroxysmal nocturnal hemoglobinuria and, occasionally, in some types of blood dyscrasia and extensive carcinomatosis. Most of these patients exhibit some degree of thrombocytopenia.

Marked, but transitory thrombocytopenia appeared, in our earlier observations(2), to be one of the characteristic features of plasma transfusion reaction. Further study, directed to investigate the specificity of this finding revealed that temporary but significant reduction in blood platelets usually followed the transfusion of compatible blood, plasma or serum of normal donors into normal recipients

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TABLE I. Thrombocytopenic Effect of the Transfusion of Blood, Plasma and Serum in Non-Thrombocytopenic Recipients (23 of 36 Cases).

Material admin.	Type and group		Diagnosis		Percentage and time of max platelet drop		
	Donor	Recipient	Donor	Recipient	%		Hr
1. Blood	O,cde	O,cde	Polycythemia vera	Normal	46	in	½
2. "	A,Cde	A,Cde	Normal	"	40		1
3. " *	O,CDe	O,CDE	"	Idiopathic hypothrombinemia	63		1
4. Plasma	O,cDe	O,CDe	"	Normal	43		1
5. "	A,CDe	A,CDe	"	"	51		2
6. "	O,CDe	O,CDe	"	Lobar pneumonia (convalescent)	54		1
7. "	"	"	"	"	48		1
8. "	"	O,CDe	"	"	51		1
9. "	"	B,CDe	"	Normal	9		½‡
10. "	O,cdE	O,Cde	"	"	45		3
11. "	O,cDE	O,CDe	"	Adenoma of prostate	50		1
12. "	O,cde	"	"	Chronic glomerulonephritis	58		1
13. "	O,Cde	O,Cde	"	Obesity	44		1
14. "	A,CDe	A,cDe	"	Cataract	40		1
15. "	AB,cDE	AB,cDe	"	Neurasthenia	56		1
16. "	B,Cde	B,Cde	"	Congenital spherocytosis§	63		½
17. "	A,Cde	A,Cde	Idiopathic hypoprothrombinemia	Peptic ulcer	60		½
18. "	"	"	Normal	Idiopathic hypoprothrombinemia	62		1
19. "	O,CDe	O,CDe	Liver cirrhosis	Iron deficiency	43		1
20. "	B,cdE	B,cDe	Disseminated lupus	Normal	30		1‡
21. "	A,cdE	A,Cde	Normal	Liver cirrhosis	48		1
22. Serum†	A,Cde	A,CDe	"	Idiopathic hypoprothrombinemia	76		6
23. "	AB,CDe	AB,CDE	"	Normal	58		3

* Transfusion followed by urticarial rash. † Transfusion followed by moderate chill and fever. ‡ Considered a negative result. § Splenectomized one year previously.

or nonthrombocytopenic patients(3). In practically all instances, no evidence of clinical reaction occurred at the time of the induced thrombocytopenia.

Materials and methods. Blood, plasma or serum were transfused into 36 compatible recipients, both normal individuals and patients suffering from various diseases but with normal platelet count. The recipients used in this series were selected from individuals who had never been previously transfused, since substances with agglutinating or lysing activity against platelets are demonstrable occasionally in the serum of individuals who had received multiple blood or platelet transfusions(4). The donors, normal subjects and a few patients with various pathological conditions but with normal platelet level, were fasting for at least 4 hours prior to donation. Four hundred ml of blood were collected in vacuum bottles containing 100 ml of ACD solution.§ The plasma was separated by centrifugation at 2,000 r.p.m. for one hour and aspirated into vacuum containers. Serum was obtained by separation from clotted blood collected in vacuum bottles; it was then kept at room temperature for 5 hours to allow complete neutralization of thrombin and stored at 4°C until used. Blood, plasma and serum were typed, grouped and cultured. They were administered to compatible recipients within 5 days of collection, when not otherwise stated, in an amount of approximately 10 (blood) or 5 (plasma or serum) ml/kg weight for adults and 16 (blood) or 8 (plasma or serum) ml/kg weight in children at a speed of 80 to 100

§ 100 ml of ACD solution (Baxter) contain: sodium citrate, 1:37 g; citric acid, 0.5 g; dextrose, 2.45 g.

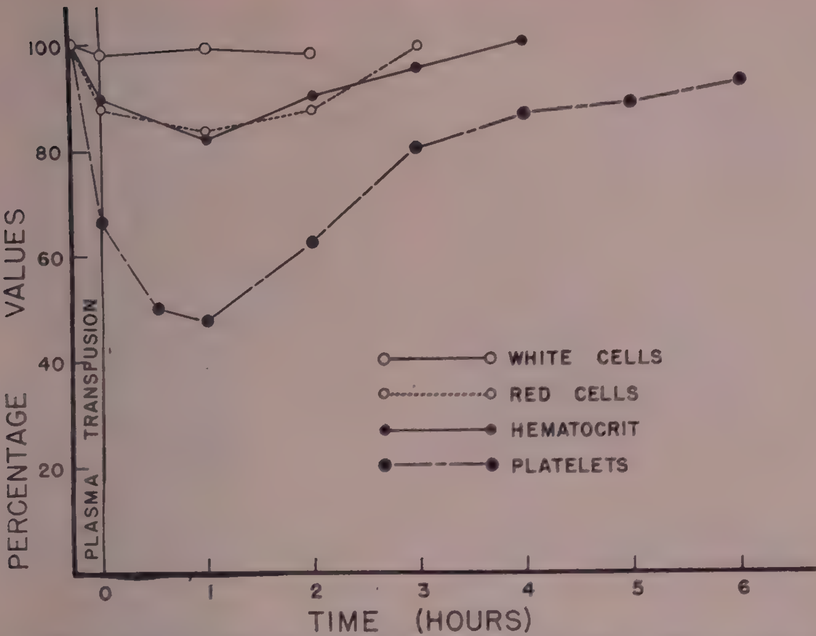

FIG. 1. Changes in hematocrit, red and white blood cells, and platelet count in normal recipients following transfusion of normal plasma (average values of 31 experiments).

drops per minute. Nineteen additional recipients received blood or variously treated plasma or plasma products, for a total of 55 observations in the course of this study. Mild clinical reactions were observed in two cases only: one recipient receiving serum (No. 16, Table I) complained of chill shortly after the termination of the transfusion and had fever for a few hours afterward; another presented urticarial rash at the end of the transfusion. Before, immediately after and at various intervals of time following the transfusion (30 minutes, 1, 2, 3, 6, 12, 24, 48 hours) several determinations were performed in all recipients. Hematocrit, red and white cell counts and differential count were obtained by standard technics(5); platelets were counted in duplicate by the indirect method of Dameshek(6). This method is accurate to ± 6.5%. The effect of the induced thrombocytopenia on the various factors and mechanisms of hemostasis was studied by means of a series of technics which have been outlined previously(7).

Results. a) *Effect of blood, plasma, and serum transfusion on the formed elements of the blood.* Moderate drop in the hematocrit reading (2 to 4 mm) for approximately 1 to 3 hours followed the transfusion of plasma (Fig. 1). At the same time red cell count and hemoglobin content decreased slightly (0.3 to 0.7 million mm^3 and 0.6 to 1.4 g per 100 ml respectively), in rough correlation with the hematocrit reading. Equivocal changes in the white cell count were noted, probably not significant in view of the daily fluctuations of this value. The number of platelets, on the other hand, dropped significantly (more than 30%) and consistently in 32 of the 36 cases studied within 3 hours of the administration of blood, plasma or serum. The percentage and time of maximum platelet drop are given in Table I which includes 23 representative cases. The lowest value was reached in most cases after 1 hour, the platelet level being back to normal as early as 1 hour and as late as 48 hours after the transfusion, with an average of approximately 4 hours (Fig. 1). The transfusion of whole blood in 3 recipients caused comparable effects, that of serum was associated with more marked and more persistent thrombocytopenia than in the case of

TABLE II. Effect of Transfusion of Normal Plasma and Serum on Platelet Level of a Normal Recipient.*

	Platelet level (per mm³)					
	Before	After	1 hr	3 hr	6 hr	18 hr
Plasma (5 ml/kilo wt)	734640	563200	374740	448000	536675	778900
Serum (" ")	751830	337740	290560	135600	293400	694400

* Plasma was injected first; patient allowed to recover completely from effect of transfusion. 48 hr later serum was injected.

blood or plasma (Table II). No hemorrhagic manifestations were observed in the course of this study. Clotting time of whole blood and native plasma in glass and Silicone-coated test tubes, clot retraction, prothrombin activity of plasma and serum were never significantly altered during the phase of induced thrombocytopenia. Bleeding time and tourniquet test remained normal. Platelets collected from the recipients at the peak of the induced thrombocytopenia appeared functionally active, since they were able to: a) restore to normal clot retraction, and b) accelerate normally the conversion of prothrombin to thrombin when added to normal native platelet-poor human plasma.

b) *Demonstration that thrombocytopenia induced by normal plasma is due to activity of a plasma component.* The administration of 100 ml of ACD solution (4 cases) and of red cells twice washed with saline solution (4 cases) was not followed by significant thrombocytopenia in normal recipients. These results indicated that the thrombocytopenia induced by transfusion was due to the activity of a factor present in plasma (and serum). *In 4 cases, however, there was no significant drop in platelet level after transfusion of the recipient's own plasma.*

c) *Thrombocytopenic effect of normal plasma after various treatments.* Five hundred ml of blood were collected from the same donors twice at an interval of 3 days. The plasma was separated, pooled and divided into 2 aliquots. The first was administered to a recipient within 2 days and the second one was given three days later, after appropriate treatment. These observations were run in duplicate, and in the second series the order of administration of the treated and untreated plasma was reversed. These experiments demonstrated that the thrombocytopenic activity of normal plasma was unaffected by storage at 4°C for 4 months, heating at 56°C for 30 minutes, decalcification by passage over a cation-exchange resin (IRC-50) (1 g per 10 ml of blood), collection in plastic bags‖ through Silicone-coated needles (which limits to a minimum the disintegration of platelets), passage through a Seitz filter. In the last experiment, since the presence of sodium citrate interferes with the removal of prothrombin by Seitz filters(8), plasma was obtained by centrifugation from blood decalcified by passage through a column of cation-exchanger IRC-50, and was subsequently filtered through Seitz under vacuum. Finally, administration of adequate amounts of heparin sodium to 4 recipients either before or during and after the plasma transfusion failed to modify the thrombocytopenic effect.

d) *Lack of thrombocytopenic activity of plasma after treatment with $Ca_3(PO_4)_2$ gel.* Under sterile conditions, 12 ml of $Ca_3(PO_4)_2$ gel 0.2 M per 250 ml of plasma to be used were centrifuged in 250 ml glass bottles at 2000 r.p.m. for 10 minutes to precipitate the solid gel and the supernatant water decanted. Since the presence of sodium citrate prevents the absorption of certain plasma proteins by gels(9), the blood was decalcified by passage through a column of ion-exchange resin IRC-50. Plasma was obtained by centrifugation, added to the gel, gel and plasma well mixed by agitation, and incubated at 37°C for 15 minutes. Plasma was then separated from the gel by centrifugation at 3000 r.p.m. for 1 hour in the cold and finally aspirated into a new 250 ml bottle. When injected into three normal recipients, the plasma so treated did not demonstrate appreciable thrombocytopenic activity (Fig. 2).

‖ Bought from Fenwalt Company, Ashland, Mass.

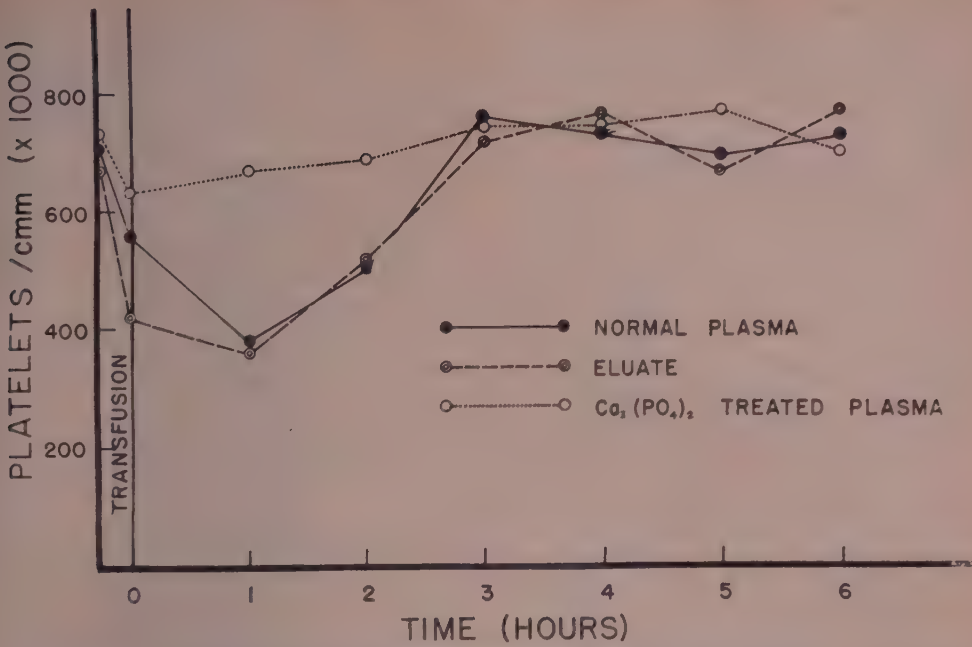

FIG. 2. Thrombocytopenic effect in normal recipients of untreated plasma, plasma treated with tricalcium phosphate gel and the eluate with sodium citrate of the material absorbed on the tricalcium phosphate gel.

e) *Elution of the thrombocytopenic factor from the $Ca_3(PO_4)_2$ gel.* The solid part of the $Ca_3(PO_4)_2$ gel was washed twice with cold distilled water, and finally treated with 15 ml of sodium citrate solution 0.2 M per 250 ml of plasma absorbed. The gel was suspended in the citrate by shaking and the bottle centrifuged in the cold at 3000 r.p.m. for 1 hour. The supernatant fluid (eluate) was separated, controlled for sterility, added to 50 ml of saline solution and injected intravenously in a normal recipient. This experiment was repeated 3 times and in every case a thrombocytopenic response practically identical to that induced by the original plasma was observed (Fig. 2).

f) *Miscellaneous observations.* No appreciable changes in number and "activity" of bone marrow megakaryocytes were observed in 2 recipients during the period of induced thrombocytopenia. Serum collected from the recipients at the peak of the induced thrombocytopenia failed to exhibit agglutinating or lytic properties against platelets, when tested with published technics(4). Samples of capillary (finger) blood and of citrated venous blood were collected from several recipients at the peak of the induced thrombocytopenia. Smears of capillary blood were prepared with Wright's stain. Plasma containing most of the platelets was obtained by centrifugation of the citrated blood at 500 r.p.m. for 5 minutes, diluted 1/20 with cold buffered saline solution and observed directly in the chamber of a hematocytometer. (All glassware, syringes and needles were coated with Silicone.) In neither case was presence of platelet clumps observed.

Discussion. The etiology of the phenomenon here described is open to many speculations. The platelet fall was clearly in excess of variations of hemodilution or of daily variations in the platelet count. Since no thrombocytopenia followed transfusion of the recipient's own plasma it is possible that the drop in platelet level might have been due to mild, sub-clinical transfusion reaction induced by a constituent present in plasma or released from the formed elements of the blood. Transfusion reactions either hemolytic

(10) or due to plasma reaction(2) are accompanied by thrombocytopenia, at times severe enough to induce hemorrhagic manifestations(10). Only 2 of the recipients included in this study, however, showed evidence of reaction, and the thrombocytopenia was, in these cases, not greater than that usually observed. As no recipient in this series had previously received blood or plasma, the possibility that thrombocytopenia might be due to sensitization to blood or plasma products, although not disproved, is rather remote. The thrombocytopenic activity of normal plasma could also have been due to incompatibility of the platelet products injected into the recipient. Although suspected, the existence of platelet groups has not been firmly established thus far. The relative uniformity of the thrombocytopenic effect (this failed to occur in only 4 of 36 recipients) seems to limit the role of platelet incompatibility in the phenomenon, but this aspect deserves further investigation.

Occasional severe prolonged thrombocytopenia, at times with occurrence of spontaneous bleeding manifestations, has been reported in normal individuals receiving plasma of patients with idiopathic thrombocytopenic purpura(11). This could conceivably be due to an excess of the thrombocytopenic factor present in normal plasma. Since platelets are destroyed at greatly increased rate in idiopathic thrombocytopenic purpura(12), one may ask whether the thrombocytopenic factor is not related to products of platelet disintegration. Serum, where most products of platelet disintegration are probably found, is in fact able to produce more sustained and pronounced thrombocytopenia (Table II). This aspect is being investigated at present, together with the possibility that the thrombocytopenic factor might be related to agents active in the process of blood coagulation, some of which exhibit similar physico-chemical properties.

The pathogenetic mechanisms through which thrombocytopenia is produced remains unsolved. Platelet production by the bone marrow megakaryocytes was not inhibited during the period of induced thrombocytopenia. The thrombocytopenic effect was of similar magnitude in 2 previously splenectomized recipients. There was no evidence of agglutination of platelets in the circulation of the recipient, nor was thrombocytopenia prevented by the administration of heparin, which decreases the agglutinability of platelets *in vivo* and *in vitro*. Platelets obtained from recipients at the acme of the induced thrombocytopenia appeared functionally active and sera, collected at the same time, did not agglutinate or lyse normal human platelets. Further investigation is in progress.

Summary. 1. Temporary, but significant thrombocytopenia was noted in 32 out of 36 individuals receiving compatible blood, plasma or serum from non-thrombocytopenic donors. No spontaneous bleeding manifestations, alterations of the various hemostatic mechanisms, changes in number or activity of the bone marrow megakaryocytes, alterations of function or morphology of the remaining platelets, agglutinating or lysing activity of the recipient's serum against normal platelets were noted. Heparinization of the recipient did not affect the thrombocytopenic effect of the transfusion of compatible plasma. 2. The thrombocytopenic effect was apparently due to a component of plasma, stable at 56°C, not absorbed by Seitz filters nor by ion-exchange resins, but absorbed on $Ca_3(PO_4)_2$ gel from which it could be eluted with sodium citrate solution. Work is in progress to establish the nature, the mechanism of action of this agent, and its relation to products of platelet destruction.

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Direct Titrimetric Method for Determination of Carbonates in Small Amounts of Serum.* (19467)

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This paper presents a simple, direct acidimetric titration procedure for the estimation of carbonates, employing small volumes of serum or plasma. A simple method employing small volumes has often posed a critical problem in the management of newborns and prematures. The principle of the new method is: 1) $HCO_3^- + Ba(OH)_2 \rightarrow BaCO_3$ (ppt.); 2) Ash to remove organic material (not necessary for pure inorganic solutions); 3) $BaCO_3 + H_3BO_3$ (hot 10% sol. in excess) $\rightarrow Ba(H_2BO_3)_2$; 4) $Ba(H_2BO_3)_2 + 2HCl \rightarrow BaCl_2 + 2H_3BO_3$; bicarbonate ion is precipitated as the barium salt, by the addition of barium hydroxide directly to the solution to be analyzed (*i.e.*, serum or plasma). The barium carbonate is dissolved in a hot solution of 10% boric acid in a manner similar to that described for dissolving calcium carbonate in the ultramicro estimation of serum calcium(1-3). This solution is diluted and titrated directly with standard hydrochloric acid to the pH of pure boric acid solution of similar strength. This titration represents the amount of carbonate in equivalents. A correction is made for the bicarbonate present in the reagents. The new method has a number of desirable features. The procedure is simple and accurate in spite of the small amounts involved. The direct titration as employed has a sharp end point. Only the stable standard acid is used (in contrast to the hitherto available titrimetric methods where serum carbonate is converted to carbon dioxide gas which is distilled into an excess of barium hydroxide, and the excess is then back titrated)(4,5). It is possible to set up simultaneously any number of determinations and complete all of them in about 1½ to 2 hours, the overall working time (with large numbers of determinations) being distinctly less than in the gasometric procedures of Van Slyke (6). Moreover, the hazard of handling large amounts of mercury is avoided and the cleaning problem is eliminated. The equipment is less expensive, and on the scale described in this paper, the method is not hinged to an ultramicro burette, which was employed, since with 0.1 N standard acid instead of 1.0 N acid one can use the usual type of two milliliter microburette. Conversely, in employing more dilute standard acids, such as 0.1 or 0.01 N, it is possible to modify this method from 0.01 ml to 0.001 ml of sample. The drawback of the new method is that it requires about 1½ hours for a single determination, which prevents it from being used in emergencies. Another drawback is that a modicum of experience must be acquired in determining the reagent blank, which in this

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method is about 2 to 5% of the total titration obtained for a normal blood serum. As regards the time factor, it is likely to be shortened in the future. Preliminary experiments indicate that further simplifications and greater speed are possible using sodium ethylene diamine tetraacetate (SED)(7) to titrate the barium in the precipitate after the barium carbonate is dissolved in dilute hydrochloric acid. It will eliminate the drying and ashing step and the solution of the barium carbonate in hot 10% boric acid, and thus a method requiring an overall time of twenty-five to thirty minutes may become feasible.

Procedure. Reagents. 1) *Saturated barium hydroxide* sol. 60 g of C.P. $Ba(OH)_2 \cdot 8H_2O$ per 1000 ml of distilled water. The solution is filtered and protected in a soda-lime tube. For the method, this solution was placed in a self-filling Machlett burette of 5 ml capacity, calibrated in 0.01 ml and protected with a soda-lime tube. The delivery arm from the upper bulb is filled with glass wool to remove any precipitate that may form. 2) *Washing fluid I.* Equal volumes of 95% alcohol and distilled water are placed in a self-filling Machlett burette of 5 ml capacity, protected with a soda-lime tube. Prior to mixing, the 95% ethyl alcohol is redistilled over barium hydroxide, and the water is boiled to remove dissolved carbon dioxide. 3) *Washing fluid II.* Previously boiled distilled water contained in a self-filling burette, protected with a soda-lime tube, or simply freshly boiled distilled water. 4) *Boric acid sol.* 10 g of recrystallized C.P. H_3BO_3 is dissolved in about 100 ml of distilled water by heating. This solution is supersaturated at room temperature, and consequently it is heated before use until the boric acid has gone into solution. The solution is used while hot. 5) *Indicator sol.* 5 parts of 0.1% Brom Cresol Green are mixed with 1 part of 0.1% Methyl Red. 0.04 ml of this indicator mixture is used for each ml of carbon dioxide free water. 6) *Standard sol.* of sodium carbonate—238.2 mg of Bureau of Standards grade Na_2CO_3 is diluted to 100 ml. 0.1 ml of this solution will evolve 0.05 ml of carbon dioxide (50 μl) measured at standard conditions. 0.1 ml = 2.248 μM. 7. *1.0 N acid* is standardized against the sodium carbonate solution using Brom Cresol Blue. 8. *Anticoagulant*—3 g of ammonium oxalate and 2 g of potassium oxalate are dissolved in 500 ml of distilled water. 0.1 ml is dried and used for each ml of blood. Instead of this mixture, 0.2 mg of heparin per ml of blood can be employed. In most cases, anticoagulant was not employed.

Apparatus. 1. *Microburette*—The Gilmont ultramicro burette was used in this study. The total capacity of this burette is 100 μl. The total volume can be delivered in steps of 1/1000 of the total so that each scale is 0.1 μl and 0.01 μl can be estimated with a fair degree of accuracy. The stirring device of the manufacturer was discarded and in its place a Spinal (Quincke) B-D Yale Luer-Lok was used. The needle point was ground flat and the Luer-Lok end was ground so as to form a round cylinder instead of the normal 4-sided appearance. This modification allows the air stream that is used for agitation during titration to be introduced to the very bottom of the tube. When an air stream is employed a soda-lime tube is introduced to remove the carbon dioxide. Other ultramicro burettes or 2 ml microburettes may be employed. When the smallest calibration of the ultramicro burrette is 0.001 ml the standard acid should be 0.1 N and when the smallest calibration is 0.01 ml, 0.01 N acid should be employed. 2. *Centrifuge tube.* This is a specially designed Pyrex glass tube with a flat bottom described by Sobel and Sobel(1). A standard 5 ml Pyrex conical tube, although less convenient, may also be employed. 3. *Stoppers.* Rubber or cork stoppers which fit the tube tightly are employed. 4. *Pipette.* 0.1 ml pipette was a capillary Mohr pipette graduated to 0.01 ml between the marks. The overall length of the graduated portion is about 140 to 150 mm. Alternately, one may employ one of the ultramicro pipettes described by Kirk(8).

Collection of specimens. 0.5 ml of whole blood is obtained without stasis and is immediately introduced under mineral oil in a 2 to 4 ml test tube. For analysis on serum the blood is permitted to clot and is centrifuged at 2000 rpm for 5 minutes to obtain the clear serum. For obtaining plasma values in these

studies, 0.2 ml of anticoagulant was dried in the tube first and the blood added and tapped to ensure adequate mixing. To eliminate water shifts, heparin may be employed as an anticoagulant. An alternate procedure of collecting specimens from the finger-tip is that described by Sobel, Besman, and Kramer(9). It must be remembered that fingertip values represent mostly arterial blood.

Procedure. 0.1 ml of serum or plasma is measured into a centrifuge tube to which is added 0.9 ml of carbon dioxide free water, and the contents are mixed by gently tapping. (Note: Always keep the centrifuge tube stoppered.) This is followed by the addition of 0.3 ml of saturated barium hydroxide. The contents of the tubes are mixed by gently tapping, and after a precipitate appears, which takes usually less than one minute, 0.5 ml of the alcohol-water washing mixture is added to each tube. The contents of the tube are mixed by gently tapping and centrifuged at 2000 rpm for 10 minutes. The supernatant fluid is decanted or aspirated off. The precipitate is mixed with the little fluid remaining at the bottom of the tube and again washed by adding 1 ml of the alcohol-water mixture and suspending the precipitate in the fluid. The contents of the tubes are centrifuged as above. The washing procedure is repeated, this time with carbon dioxide free water. (This last washing was found to be empirically better than the alcohol-water washing because it removes the last traces of precipitating agent.) After centrifuging, as much of the supernatant fluid is removed as is feasible without disturbing the precipitate, and the tubes are placed in an air oven at 110° to 120°C until dry (about 20 minutes), and in a muffle furnace to incinerate organic matter adsorbed to the precipitate. (This step is not necessary for inorganic solutions.) After removal from the furnace, the tubes are allowed to cool. They are then immersed in a small boiling water bath, and to each tube 0.2 ml of hot 10% boric acid is added. After one to 2 minutes each tube is tapped to assist in the solution of the barium carbonate. The solution containing the dissolved precipitate is diluted with 0.8 ml of distilled water containing the indicator, and allowed to cool to room temperature. It is then ready for titration with an ultramicro burette. The tip of the burette is immersed below the surface, while a gentle stream of nitrogen or air is bubbled through. The solution is titrated back to the pH of a pure boric acid solution of similar strength. A titration blank containing only the boric acid and the indicator solution is used as the color matching tube. The blue to pink end point is very sharp and a sensitivity to 0.00001 ml can be obtained. This corresponds to an ability to detect 0.05 meq/liter of carbon dioxide. Several reagent blanks are run through with each determination. The value is subtracted from the values obtained on the unknown specimen.

TABLE I. Influence of Dilution on Completeness of Precipitation.

Specimen (.1 ml)	H_2O,* ml	Carbonate precipitated, vol %	Van Slyke value, vol %
Serum I	.1	39.1	63.5
	.4	51.4	63.5
	.9	63	63.5
Standard $NaHCO_3$ sol.†	.1	50.1	50.1
	.4	50.4	50.1
	.9	50.7	50.1

* The administration of water was followed by .3 ml of saturated $Ba(OH)_2$ and 1 ml of alcohol-water mixture, and treated as described under Procedure.

† Theoretical = 50 vol %.

TABLE II. Influence of $Ba(OH)_2$ Concentration on Completeness of Precipitation.

Final conc. of $Ba(OH)_2 \cdot 8H_2O$	Authors' method, vol % Serum 1	Serum 2	Na_2CO_3 standard
1	41.2	52	50.1
1.4	72	63.2	50.6
2	72.7	63.9	51.5
2.5	73	64.3	51.7
Value by method of Van Slyke, *et al.*	72.3	63.4	50.2

Calculations. 1 μl of 1 N acid = 0.5 μE of bicarbonate, or 11.13 μl of carbon dioxide. If 0.1 ml of blood serum is used, then meq/L = μl of titration x 5, and volume % = μl of titration x 11.13.

Discussion of procedure and results. As is evident in Table I, preliminary dilution of plasma or serum is necessary for complete precipitation, although this is not the case for

TABLE III. Comparison Between Authors' Titrimetric Method and the Manometric Method of Van Slyke, in 30 Patients.

Diagnosis	Condition of sample	Authors' method CO_2, vol %	Van Slyke CO_2, vol %	% of Van Slyke value
Coronary	Clear	55.3	56.5	98
Post op. hernia	"	60.3	58.4	103
Coronary	"	68.8	66.6	103
Pleurisy	"	62.8	62.8	100
Chronic pancreatitis	"	62	60.7	102
Urethral growth	"	58.5	58	101
Cirrhosis	"	54.7	54.8	100
Malignancy	"	65	68.1	96
Thrombophlebitis	"	65	64.8	100
Possible uremia	"	48.3	48.6	100
Coronary	Chylous	64.5	62.7	103
Vaginal bleeding	Clear	59.5	59.5	100
Cholecystitis	"	55.1	53.5	103
Incomplete abortion	"	58	58.2	100
Coronary	"	62.8	62.5	101
Coronary	"	65.6	65.8	100
Fever of unknown origin	"	62.2	62.4	100
Coronary	"	57.6	57.6	100
Bleeding ulcer	Hemolyzed	59	60.5	98
Coronary	Clear	59.5	61.8	97
Acidosis	"	25.5	25.9	98.5
Undetermined	"	59.3	60	99
"	"	45.5	45.5	100
"	"	41	42	98
"	"	50	49.1	101
"	"	41.8	41.9	100
"	"	51.1	50.7	100
"	"	58.8	59	100
"	"	44.4	44.2	100
"	"	52.2	52.3	100
	Mean	55.80	55.81	100.05
				Std. dev. $\pm$ 1.698
				Std. error $\pm$ .3100

pure inorganic solutions. The explanation may be that some of the carbonate is protein bound. This point may require further study, and the difference between carbonate that can be precipitated in concentrated serum, as compared to that in diluted serum, may be a measure of some deviation from the normal in clinical conditions.

Conditions of precipitation. In precipitating the barium carbonate, at first an attempt was made to follow the conditions described by Exton *et al.*(10), as the first step in the precipitation of serum carbonate. However, under these conditions the precipitation of barium carbonate was only 60% to 70% complete in the absence of Celite which these authors employed. A systematic investigation, shown in Table II, indicated that when the concentration of $Ba(OH)_2 \cdot 8H_2O$ in the reaction mixture is about 1.4%, the serum carbonate is quantitatively precipitated. This concentration was therefore chosen for the conditions of precipitation, since any further increase would simply increase the reagent blank, and much below this level, the precipitation is incomplete under the conditions presented. It must be pointed out that the concentration of barium hydroxide in Table I is given prior to the addition of the alcohol and water. The addition of the alcohol-water mixture following barium hydroxide was introduced because it was observed that the results with serum are somewhat lower even though in standard solutions precipitation was complete. The alcohol may accomplish a dual purpose, that is, decrease the solubility of barium carbonate as well as assist in the decomposition of protein bound carbonate. The conditions for washing were arrived at empirically, and it is by no means certain that

further investigation may not produce alternate procedures equally satisfactory.

Results. Table III presents a series of analyses on blood serum taken under oil, compared to results on the manometric method of Van Slyke *et al.*(6). The mean result is 100.05% of the Van Slyke value with a standard deviation of ± 1.70 and a standard error of 0.31. Thus, it is evident that the present method yields a fairly high degree of precision even though carried out on one-tenth of the amounts that the Van Slyke method employs.

Summary. 1. A direct acidimetric titration method is presented for the estimation of serum carbonate in small volumes of serum or plasma. Serum bicarbonate is precipitated as the barium salt by the addition of barium hydroxide, the precipitated bicarbonate is dissolved in hot boric acid, and the $Ba(H_2BO_3)_2$ in solution is titrated with standard acid to the pH of a pure solution of boric acid with the aid of an ultramicro burette. The titration represents the bicarbonate in equivalents. 2. Various critical points in the method were investigated and are discussed. The probable existence in serum of a protein bound carbonate is indicated. There is close agreement between the values obtained in this method on 0.1 ml of serum and the results obtained by the manometric procedure of Van Slyke, employing 1.0 ml of serum.

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Comparative Studies of the β Disturbance of Electrophoretic Patterns in Disease. (19468)

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In electrophoretic patterns of the descending boundary of plasma or serum obtained by the Schlierin scanning technic, the β globulin component often contains a sharp spike extending upward. In an electrophoretic study of the serum from poliomyelitis patients, Kelley and coworkers observed a definite decrease in the length of this β spike(1,2).

In recent studies on poliomyelitis in this laboratory(3), the decrease of the length of the β spike in plasma and serum patterns was compared with those from normal individuals. Since the results, especially those from normal individuals, did not agree with those reported by Kelley *et al.*(2), it was decided to compare the length of the β spike of electrophoretic patterns of plasma and serum from several diseases.

TABLE I. The Beta Spike of Electrophoretic Patterns of Plasma.

Disease	No. of samples	Ratio of beta spike to albumin peak, %	Change in length of beta spike, % None or slight	Marked
Normals—children	77	42.4	39	61
" —adults	37	46.2	45.9	54.1
Poliomyelitis	134	36.4	37.3	62.7
Rheumatic fever	224	41.3	36.2	63.8
Liver disease	142	34.2	36.6	63.4
Diabetes	202	31.4	16.3	83.7
Arthritis	134	42	44	56
Arthritis before ACTH or cortisone	19	24.5	15.8	84.2
Arthritis after ACTH or cortisone	24	51.1	41.6	58.4

Experimental. The plasma and serum samples were obtained from normal children, normal adults and from patients with various diseases. These samples were diluted with 3 volumes of a barbiturate buffer, pH 8.6, ionic strength 0.1, and dialyzed at 2 to 6°C for 3 days with daily change of buffer. Electrophoresis was carried out for 120 minutes in the Longsworth modification of the Tiselius apparatus using the analytical cell. The electrophoretic pattern of the descending boundary was used for the measurement of the length of the β spike. Complete electrophoretic data on the plasma and serum patterns from many of the subjects in this paper have already been reported in previous publications from this laboratory.

Kelley and coworkers graded their patterns as follows: 0 or no change from the normal, where the β spike was long and narrow and extended upward to the full length of the albumin peak. 1+ indicated a shortening and broadening of the β spike with the length equal to about 2/3 that of the albumin peak. 2+ indicated a marked decrease of the β spike to approximately 1/3 the length of the albumin peak. 3+ indicated complete absence of the β spike.

An attempt was made to devise a more quantitative comparison of the β spikes of the various electrophoretic patterns. For this purpose, the albumin peak and β spike of each pattern were measured. A ratio representing the length of the β spike expressed as a percentage of the length of the albumin peak was then calculated. Values obtained in this fashion were compared with the Kelley system of rating. Since a range of values was necessary for accurate comparison, these are also shown below.

0 = 100% (of the length of albumin peak) or a range of 83.3 to 100%
+1 = 66.6% or range of 50 to 83.3%
+2 = 33.3% or range of 16.6 to 50.0%
+3 = 0 or range of 0 to 16.6%
No or slight change, 50 to 100%
Marked change, 0 to 50%

Results. Employing the rating system outlined above, the length of the β spikes of approximately 1100 plasma and 500 serum patterns were examined. Table I represents the results obtained from the plasma samples. A comparison of the ratio of the length of the β spike to the length of the albumin peak of these patterns shows that plasma from normal children or adults exhibits a ratio similar to those from patients with various diseases. Although the average length of the β spike in disease was shorter than that from normal individuals, this difference was not as marked as that in the serum samples reported by Kelley and coworkers. An interesting observation was the marked increase in the ratio observed in a small group of arthritis patients after treatment with ACTH or cortisone.

Kelley *et al.* designated a change in the length of the β spike as "no or slight" if the pattern was judged 0 or +1. They called a change "marked" if the pattern was judged +2 or +3. For comparative purposes, our patterns were judged as previously described

TABLE II. The Beta Spike of Poliomyelitis Plasma Patterns.

Patient classification	No. of samples	Ratio of beta spike to albumin peak, %	Change in length of beta spike, % None or slight	Marked
All patients	134	36.4	37.3	62.7
Spino-bulbar	22	37.8	40.1	59.1
Spino-bulbar, died	13	27.4	30.8	69.2
With paralysis	17	38.7	35.3	64.7
With weakness	23	37.8	30.5	69.5
Abortive or non-paralytic	72	35.7	37.5	62.5

TABLE III. The Beta Spike of Electrophoretic Patterns of Serum.

Disease	No. of samples	Ratio of beta spike to albumin peak, %	Change in length of beta spike, % None or slight	Marked
Normals—children	19	29.3	21.1	78.9
" " Kelley *et al.*	20	—	80	20
Normals—adults	24	36.2	29.2	70.8
Poliomyelitis	47	38.3	34.1	65.9
" Kelley *et al.*	142	—	50	50
Rheumatic fever	132	35.7	24.2	75.8
Liver disease	80	21.6	20	80
Diabetes	54	27.6	18.5	81.5
Arthritis	72	48.2	52.8	47.2

and divided into those showing marked change and those showing no or slight change. These values are shown in Table I and exhibit marked similarity, with the exception of patterns from diabetes and the small group of arthritis patients before treatment with ACTH or cortisone.

A similar study of the β spike in plasma samples from poliomyelitis is shown in Table II. The classification of patients is similar to that previously reported from this laboratory(3). Kelley and coworkers were unable to observe a correlation between the change in the length of the β spike and the age and sex of the patient, the extent of paralysis, the occurrence of bulbar symptoms or the temperature and pulse of the patient at the time the blood sample was obtained. The only marked difference in the ratio of the β spike to the albumin peak was exhibited by the patients with spino-bulbar poliomyelitis that died. Also, this group and the group of patients that exhibited weakness had the highest percentage of marked changes in the β spike.

Table III presents a study of the changes in the length of the β spike in serum samples. The ratio of the β spike to the albumin peak in these patterns shows similar values for normal individuals and patients with various diseases. The difference in our results and those obtained by Kelley *et al.* is clearly shown in the last 2 columns in Table III. Kelley's value for normal children shows a high percentage of patterns with long β spikes which would result in a very high ratio of the β spike to the albumin peak. Our values for normal children and adults almost reversed those of Kelley *et al.* The changes in length of the β spike shown by our patients with disease are, in general, more marked than those reported by Kelley and coworkers in poliomyelitis. Patients with arthritis were the only ones that exhibited patterns similar to those of Kelley *et al.*

Discussion. The essential difference between our results and those reported by Kelley and coworkers is the length of the β spike in electrophoretic patterns obtained from normal individuals. Apparently a β spike which extends upward to the full length of the albumin peak is not characteristic of a normal

individual or of a patient with disease. The appearance and length of the β spike depends somewhat on the dietary state of an individual, the amount of fat present in the blood, and the time of withdrawal of the blood sample. It is interesting to note that in the classical electrophoretic patterns reported by Longsworth and coworkers the β spike was evident in most patterns and was long and narrow(4). On the other hand, patterns reproduced in more recent publications very often show a very short β spike or in some cases the complete absence of this spike. It is also interesting to observe that in the present investigation plasma and serum samples from normal children exhibit shorter β spikes than those from normal adults.

Before drawing any definite conclusions as to the cause of the slight differences between the length of the β spike in patterns from normal individuals or those from patients with disease, the origin of the β spike must be determined. We plan to extend this investigation to include a study of the origin of the β spike in electrophoretic patterns of plasma and serum.

Summary. 1. Electrophoretic patterns of approximately 1100 plasma and 500 serum samples have been examined for changes in the length of the β spike. These samples were obtained from normal children and adults and from patients with poliomyelitis, diabetes, rheumatic fever, arthritis, and liver disease. 2. Minor changes occurred in the average length of the β spike in electrophoretic patterns from plasma and serum when patients with disease were compared with normal individuals. These changes were also observed when patterns from patients with one disease were compared to patterns from other diseases. These changes were not marked enough to differentiate between diseases or between normal individuals and those with disease.

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Effect of Lyxoflavin on Growth of Baby Pigs Fed a Synthetic Diet. (19469)

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In 1949 Pallares and Garza(1) isolated from human heart myocardium a pentoseflavin which they identified as l-lyxoflavin. The significance of the presence of lyxoflavin in human heart muscle was not discussed by these investigators. However, Emerson and Folkers(2) have suggested the possibility that lyxoflavin may be a new member of the vit. B complex. Lyxoflavin differs from riboflavin only in the configuration of the groups about carbon 4 of the pentose side chain.

The present experiments were designed to study the effect on growth of feeding lyxoflavin to baby pigs on a "synthetic milk" ration containing all of the known nutrients.

Experimental. Baby pigs from one to 2 days of age were used as experimental animals. The technic of feeding and care of the animals has been described by Johnson *et al.*(3). The composition of the basal rations used in these experiments is given in Table I. In addition

TABLE I. Composition of Basal Diets.

	Casein diet, %	Alpha-protein diet, %
Casein (vit.-free)	25	—
Alpha-protein	—	24.6
DL-Methionine	—	.4
Sucrose	68	68
Cottonseed oil	1	1
Minerals	6	6

The above materials were made into a "milk" containing 19.5% solids.

Vitamins* added/liter "synthetic milk"

	mg
Thiamine hydrochloride	1
Riboflavin	2
Pyridoxine hydrochloride	2
Ca pantothenate	12
Nicotinic acid	4
Inositol	40
Choline	400
Biotin	.016
Ascorbic acid	.016
Folic acid	.08
2-Methyl-1,4-napthoquinone	.4
Alpha tocopherol acetate	1.54
Vit. A	3000 I.U.
D_2	300 I.U.
B_{12}	.8 μg/kg body wt /day, given by inj.

* Thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, Ca pantothenate, nicotinic acid, biotin, ascorbic acid, crystalline vit. B_{12}, alpha tocopherol acetate, choline chloride and synthetic L-lyxoflavin were supplied by Merck and Company, Inc., Rahway, N. J., through the courtesy of Dr. D. F. Green and Dr. Karl Folkers. Folic acid was supplied by Lederle Laboratories, Inc., Pearl River, N. Y., through the courtesy of Dr. T. H. Jukes.

the pigs were given protamone at a level of 0.01% of the dry matter of the ration in order to enhance the development of a deficiency state by increasing the metabolism of the animal. In a preliminary experiment it was found necessary to substitute "milk" containing 30% of lard for the low fat milk during the first few days. The amount was gradually decreased so that by the end of the first week the pigs were receiving the "synthetic milk" as described in Table I. In a preliminary experiment protamone was fed at a level of 0.1% of the dry matter of the diet. This level of protamone resulted in a high mortality rate and the level was therefore reduced in the subsequent experiments.

A summary of the total performance of the pigs in Exp. 1 and 2 is shown in Tables II and III. In Exp. 1 the pigs received the casein basal ration for 49 days. Group 2, which received lyxoflavin supplementation (4 μg per g of dry matter), outgained the control group at a highly significant rate on significantly less dry matter consumed per kg gain in body weight. Because of the loss of several pigs in this experiment due to the physical nature of the diet, alpha-protein was substituted for the casein in Exp. 2. The data of this experiment are given in Table III. Using the analysis of covariance (Fisher(4), and Snedecor(5)), the difference in weight gains brought about by the addition of 4 μg of lyxoflavin per g of dry matter consumed was found to be very highly significant ($P < 0.001$).

To study the presence of lyxoflavin in urine or tissues a differential assay procedure was developed by assaying standard riboflavin and standard lyxoflavin solutions and mixtures of the 2 both microbiologically and fluorometrically. For the fluorometric assay the solutions were adsorbed on florisil and the riboflavin and/or lyxoflavin was eluted by passing 20 to 23 ml of a solution of 20% pyridine in 2% acetic acid through the column. The eluate was made up to 25 ml. To an aliquot of the eluate was added 2 drops of glacial acetic acid and 2 drops of 4% potassium permanganate, and the solution was swirled and left to stand for 2 minutes. The solution was then cleared with 3 to 4 drops of 3% hydrogen peroxide and the fluorescence determined in a Coleman 12A fluorophotometer. A dilute solution of sodium fluorescein was used as primary standard. The results of a series of fluorometric

TABLE II. Response of Baby Pigs to Lyxoflavin (Exp. 1).

Items compared	Group 1 basal	Group 2 basal + lyxoflavin
No. of pigs	3	4
Avg initial wt, kg	1.91	1.82
" final " "	18.25	19.96
" total gain "	16.34	18.14*
" daily food consumed, kg	.52	.53
" food consumed (per kg gain)	1.56	1.43†

* Highly significant over Group 1 ($P = < .01$).
† Significant over Group 1 ($P = < .05$).

TABLE III. Response of Baby Pigs to Lyxoflavin (Exp. 2).

	Group 1 basal	Group 2 basal + lyxoflavin
No. of pigs	4	5
Avg initial wt, kg	1.46	1.41
" total gains (8 wk)	14.73	16.40*
" food consumed (per kg gain)	1.72	1.64

* Significant over Group 1 ($P = < .001$).

TABLE IV. Lyxoflavin and Riboflavin Excretion in Urine of Pigs (mg per Day).

Group No.	No. of pigs	Lyxoflavin intake	Riboflavin excreted*	Riboflavin +lyxoflavin excreted†	Lyxo-flavin excreted‡	% lyxoflavin intake excreted in urine
I Exp. 1	2		2.3	2.4		
2	1		2.5	2.6		
Avg both exp.			2.4	2.5		
II Exp. 1	3	2	2.3	3.1	1.2	60
2	5	2.7	2.75	3.9	1.8	66
Avg both exp.		2.4	2.57	3.6	1.58	65.8

* By *L. casei* assay. † By fluorescence assay.

‡ Difference between casei assay and fluorescence assay multiplied by 100/58.7 and corrected for the 106 (avg) μg per day higher riboflavin excretion by fluorometric assay of the Group I pigs.

readings gave values for the lyxoflavin of 55.5 to 60.4% (average 58.7%) of the riboflavin reading at that same concentration.

A microbiological assay on these standard solutions was carried out simultaneously using *Lactobacillus casei* as the test organism. From the results of this assay it was apparent that lyxoflavin did not replace riboflavin in the growth of this organism.

The lyxoflavin content of the unknowns may then be calculated as follows:

$$\frac{(\text{Fluorometric assay value}) - (\text{microbiological assay value}) \times 100}{58.7}$$

In order to determine whether riboflavin may be converted into lyxoflavin in certain tissues of the body, homogenates of liver and heart were incubated in phosphate buffer in the presence of 50 μg of added riboflavin per sample (1-2 g) of tissue. The solutions were incubated at 37°C under toluene for 4, 8, 16, and 24 hour periods. At the conclusion of the incubation periods, the riboflavin content was determined both fluorometrically by the method of Hodson and Norris(6) and microbiologically by the *Lactobacillus casei* method of Snell and Strong (as given by Johnson (7)). No change in riboflavin content and no evidence of lyxoflavin formation was found following incubation.

Limited data on the lyxoflavin content of heart muscle indicate the possibility of some lyxoflavin being stored in this organ. Using the average values obtained it was found that the hearts from the lyxoflavin-fed pigs averaged 0.60 μg per g higher in riboflavin content by fluorometric assay than by microbiological assay. On the other hand the heart tissue of the pigs which did not receive lyxoflavin averaged only 0.175 μg per g higher in riboflavin content by fluorometric assay than by microbiological assay. If one assumes this 0.175 μg to be due to fluorescent substances other than lyxoflavin there is still 0.425 μg per g unaccounted for in the heart tissue of lyxoflavin-fed pigs which may be due to lyxoflavin. Since it was found that lyxoflavin will fluoresce only 58.7% as much as riboflavin, one may calculate a lyxoflavin content of approximately 0.7 μg per g in the heart tissue.

Twenty-four hour urines were collected in both experiments. The urine was analyzed for lyxoflavin using the same differential assay. Paper strip chromatograms were also run on the urine. The data on urinary excretion of lyxoflavin are presented in Table IV. An average 68.1% of the lyxoflavin intake was accounted for in the urine by this procedure after correcting for interfering fluorescent substances. Sixteen different solvent systems were used in an attempt to separate riboflavin and lyxoflavin on a paper strip chromatogram. None of the solvent systems tried separated these two closely related compounds. However, paper strip chromatograms of the urine of pigs fed lyxoflavin, using a solvent system of butanol, pyridine, and water, showed a violet spot which was not present in the urine of pigs fed the basal ration only. This metabolite differed from both lyxoflavin and riboflavin.

Discussion. Emerson and Folkers(8) have

reported an increased rate of gain in rats fed lyxoflavin as compared to the basal group. From the results of the experiments reported herein, it appears that lyxoflavin also has growth promoting activity in baby pigs when the basal ration contains a minimum amount of fat and contains protamone to enhance the deficiency state.

It appears possible that lyxoflavin may be a new member of the vit. B complex with a biological role different from that of riboflavin and other known vitamins. However, the fact that the baby pigs do quite well on the basal ration which is devoid of any known lyxoflavin might indicate that lyxoflavin exerts its effect in a manner similar to that of the antibiotics or of the surfactants(9) or other growth stimulants, rather than due to the vitamin nature of the compound. The high amount of the lyxoflavin fed which was excreted in the urine might indicate tissue saturation and implies that a lower level would have been as effective as the level used. In the case of riboflavin approximately 30% of an intake of 11 μg per g was excreted in the urine as compared to 68.1% of 4 μg per g for lyxoflavin.

Lyxoflavin is apparently not converted into riboflavin in the animal body as Emerson and Folkers(8) report that synthetic lyxoflavin is devoid of riboflavin activity in rats by the standard assay. We have confirmed this by *in vitro* studies and have also confirmed their report that lyxoflavin is inactive for *Lactobacillus casei*.

It appears that some lyxoflavin may be stored in the heart, however, the amount is small and with a differential assay it is more difficult to determine these minute quantities than with a direct assay.

Summary. 1. The addition of 4 μg of synthetic l-lyxoflavin per g of dry matter consumed in 3 experiments significantly increased the rate of gain and efficiency of feed utilization of baby pigs fed a low fat basal ration including 0.01% protamone. 2. Using a differential assay procedure for lyxoflavin it was found that approximately 67% of the lyxoflavin intake was excreted in the urine. 3. *In vitro* studies of heart and liver tissue did not indicate any conversion of riboflavin into lyxoflavin.

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Shwartzman Phenomenon II. Suppressive Action of HN_2 on Antigen-Antibody Provocation. (19470)

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The basic experiment demonstrating the phenomenon of local tissue reactivity (Shwartzman phenomenon) consists of the intradermal injection of a small amount of a suitable bacterial filtrate (preparatory injection), followed after some 24 hours by an intravenous injection of a quantity of the same material (provocative injection). In susceptible rabbits, a zone of hemorrhagic necrosis appears at the site of the preparatory injection 3 to 5 hours after the intravenous injection of filtrate(1). The phenomenon can

also be elicited when the intravenous injection of bacterial filtrate is replaced by a challenging intravenous injection of antigen into a sensitized animal (antigen-antibody reaction *in vivo*)(1). The mechanisms through which these agents provoke the phenomenon seem to be different, at least in part, since Shwartzman has shown that ACTH strongly suppresses the reaction of hemorrhagic necrosis when the phenomenon is provoked by an intravenous injection of a bacterial filtrate (2), but has no effect when an antigen-antibody reaction *in vivo* is substituted for the latter(3).

The reported contrast in the reactions to the provocative agents in rabbits treated with ACTH prompted a similar comparison when a nitrogen mustard is used as the suppressive agent. Methyl bis β-chloroethyl amine hydrochloride has been shown by Becker to suppress completely the reaction of hemorrhagic necrosis when 2 mg/kg are given intravenously 4 days before the intravenous provocative injection of bacterial filtrate(4). The observation has been amply confirmed (5,6), but in none of the studies on mustard suppression reported heretofore has the antigen-antibody reaction *in vivo* been used to provoke the phenomenon.

Methods and materials. Rabbits of both sexes and of various strains, weighing 1800 to 3000 g were used. The animals were maintained in separate cages on routine laboratory feed and water *ad lib*. *Skin preparation.* The abdominal skin of the rabbit was lathered and shaved just prior to the preparatory intradermal injection. The preparatory injection consisted of 0.5 ml of *Meningococcus 44B* filtrate,* 0.5 ml of a filtrate of *E. coli*, or 0.25 mg of a partially purified polysaccharide of *S. typhimurium*. The injections were made about 24 hours before the challenging intravenous antigen injection and the preparatory substances were varied to obviate acquired immunity when repeated experiments were done on the same animal. *Nitrogen mustard.* Methyl bis β-chloroethyl amine hydrochloride, (HN_2) was injected intravenously. Solutions were freshly prepared before each group of injections and were made up to 1 mg/ml in isotonic saline or distilled water. Two mg/kg of the agent were injected 4 days before the challenging intravenous injection of antigen. *Antigen-antibody reactions.* One or two sensitizing injections of 2 ml of fresh human serum were given intravenously to each rabbit. An interval of 7-10 days elapsed when two sensitizing injections were used. Seven to ten days after the last sensitizing injection, and 24 hours after the intradermal preparatory injection, a challenging injection of 2 ml of serum was given. Small groups of rabbits served alternately as mustard-treated and control subjects, resting 10-30 days between experiments. Each group was used 2 or 3 times.

* Kindly supplied by Dr. G. Shwartzman.

TABLE I. HN_2 Suppression of Shwartzman Phenomenon Provoked by Antigen-Antibody Reaction *in vivo*.

	No. of exp.	Pos.	Neg.
Controls	19	14	5
HN_2-treated	15	0	15

Results. The results of the experiments are summarized in Table I.

Conclusion. It is evident from the data presented that HN_2 completely suppresses the reaction of hemorrhagic necrosis when an attempt is made to provoke the Shwartzman phenomenon by an antigen-antibody reaction *in vivo*.

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Toxicity of Progesterone in the New-Born Mouse.* (19471)

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During a systematic study of the effect of adrenocortical and related steroids on the new-born mouse, progesterone, a relatively innocuous steroid in mature mice, was found to be unusually toxic. Symeonidis(1,2) had previously shown that mice and rats were remarkably susceptible to progesterone during the last trimester of pregnancy; progesterone inducing fetal death with resorption or stillbirths and, in some instances, the mothers died with histological changes suggestive of eclampsia. It was, therefore, of interest to determine the toxicity of progesterone in new-born mice, and to find the age at which the mice developed tolerance to this steroid.

Materials and methods. A variety of pure mouse strains and hybrid mice were used. Pregnant mice were placed in separate cages during the last few days of pregnancy, and examined daily. The mice usually littered during the night, and the new-born found each morning were classified as less than 24 hours old. The mice were divided into age groups of 1) less than 24 hours, 2) 25-48 hours, 3) 49-72 hours, 4) 73-120 hours, 5) 121-168 hours, for injection. Mice in each litter were divided usually into a control and a treated group. Injections of progesterone† were made with a No. 25 needle under the skin of the back, and a special effort was made to prevent leakage. The usual volume was 0.05 cc, but in older mice 0.10 cc was given. The control mice received an equivalent volume of physiological saline. The mice were examined and weighed daily, and usually discarded 10 days after injection. A total of 89 litters consisting of 488 new-born mice was used. In order to confirm the reported toxicity of progesterone during the last third of pregnancy, conspicuously pregnant mice (usually 16-18 days of gestation) were injected with progesterone subcutaneously, and the size of the fetuses and the delivery date were used as evidence for the age of the fetuses when the mother was injected.

Results. Suckling mice. Table I summarizes the toxicity data on the mice injected with progesterone and their litter mate controls. The new-born mice were unusually susceptible to progesterone, with the LD_{50} estimated at 0.1 mg/mouse. Mice obtained immediately after delivery succumbed to 0.02 to 0.05 mg/mouse. It was often difficult to draw definite conclusions from the work with very young mice, since disturbing the litter or intoxicating half the litter sometimes alienated the mother, so that the litter was not cared for and all the mice died, including the untreated ones. Results could be regarded as significant only when the control mice survived. The tolerance of the mice to progesterone increased rapidly, as shown in Fig. 1, so that at 72 hours the LD_{50} was about 20 times the 24-hour figure. Resistance then developed at a relatively slower rate, but at 7 days the LD_{50} dose on a mg/kg basis showed a 40-fold increase over the LD_{50} dose in the new-born mouse.

Following a dose in the range of the LD_{50}, the progesterone-treated mice became lethargic, feeble and failed to nurse. The mice injected soon after birth died within 24 hours or survived, whereas the older mice were sometimes prostrated and continued to lose weight for 3-4 days before death. Occasionally the seriously intoxicated mouse recovered after 2 to 3 days and began to nurse and gain weight.

Progesterone appeared to be somewhat spe-

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† Progesterone was made available through the courtesy of the Schering Corp. and was prepared in aqueous suspension of 200 mg/cc; this was appropriately diluted with saline before use.

TABLE I. Approximate LD_{50} of Progesterone in Suckling Mice at Various Ages.

Age at inj., hr	Dose, mg/mouse	Treated: No. inj. Litters	Treated: No. inj. Mice	Treated: Avg wt at inj., g	Treated: % survival, 5 days	Control: No. mice treated	Control: % survival, 5 days	Estimated LD_{50} of progesterone: mg/mouse	Estimated LD_{50} of progesterone: mg/kg
0–24	.05	2	6	1.4	100	6	100	.1	70
	.10	9	29		52	26	77		
	.20	5	17		12	10	90		
	.5–1	4	12		0	15	100		
25–48	.30	2	10	1.7	90	—	—	.4	235
	.50	6	19		38	15	94		
	1	3	11		18	5	60		
	1.5–2	3	9		0	6	100		
49–72	1	4	10	2.1	90	7	72	2.5	1200
	2	9	25		80	17	88		
	3	4	12		25	6	100		
	5	2	6		0	6	66		
73–120	2	1	3	2.5	100	3	100	4	1600
	5	10	32		22	3	100		
	10	4	12		0	6	100		
121–168	5	3	9	3.3	78	9	100	9	2700
	10	15	104		45				
240	10	3	22	4.8	91	—	—	>10	>2100

cific in inducing acute toxicity in new-born mice, since the injection of 1 mg doses of cortisone acetate, 17 hydroxy-pregnenolone, desoxycorticosterone acetate and Δ^5 pregnenolone did not induce acute deaths regularly during the first 24 hours after birth. The activity of these steroids can not be compared quantitatively with that of progesterone, since they were each suspended differently in saline, and slower absorption from the injection site probably diminishes their acute toxicity.

Pregnant mice. Five mg of progesterone injected subcutaneously during the 16th to 20th day of gestation caused fetal death in 6 of 8 mice, and 10 mg given to 2 mice caused fetal death in both cases. This observation confirms the detailed report by Symeonidis(1).

Discussion. Symeonidis(2) has presented the following possible explanations for the lethal effect of progesterone on the fetus and occasionally the mother during the last third of pregnancy in the mouse and rat: 1) progesterone induces a hormonal imbalance, 2) it directly damages the embryo and placenta, resulting in substances toxic to the mother, 3) it has a direct toxic action on the maternal tissues. The marked susceptibility shown by the new-born mouse to progesterone may be interpreted as supporting the second hypothesis, that progesterone is acting directly on the fetus in causing fetal death, and maternal toxicity is secondary to fetal injury. A dose of 5 mg of progesterone in a 35 g pregnant mouse, if evenly distributed, would give a concentration of 0.15 mg/g of tissue, which is well above the dose that would be lethal if injected into the new-born mouse. Five mg of progesterone, when given during the first and second thirds of pregnancy is not injurious to the fetus or mother, indicating that the susceptibility to progesterone appears during an advanced stage of embryonic development.

The mechanism whereby the new-born mouse develops resistance to progesterone shortly after birth is not known. Selye(3) has found that certain steroids have anesthetic properties in the rat, and this activity is enhanced by partial hepatectomy. Progesterone exhibited a high degree of anesthetic activity by this test. It seems reasonable to suggest that the toxic action of progesterone in the new-born mouse is due to its anesthetic action, as indicated by the behavior of the mice. As specific systems develop in the mouse, possibly in the liver, the ability of the mouse to cope with progesterone rises rapidly. The

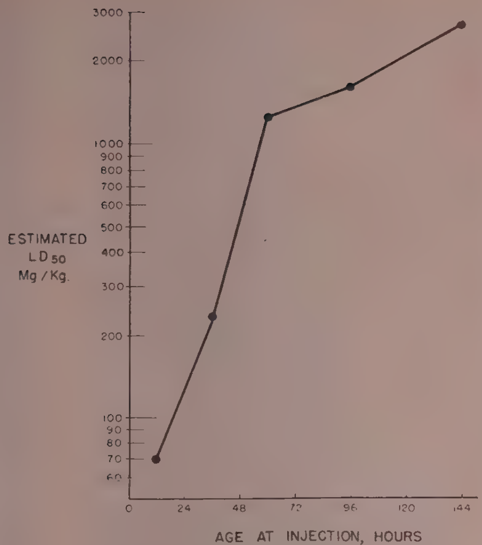

FIG. 1. Rise in tolerance of the baby mouse to progesterone with increasing age.

method of progesterone detoxification and catabolism is poorly understood(4). The data obtained on the development of resistance to progesterone within 2 to 3 days after birth may lead to an understanding of the mechanism whereby progesterone is inactivated or excreted.

Summary and conclusions. The new-born mouse is highly susceptible to the toxic and lethal action of progesterone but, within 2 to 3 days, resistance develops rapidly, and by the 7th day the mouse can tolerate about 100 times as much progesterone as when one day old. Closely related steroids, at equivalent and even higher doses, do not cause the acute lethal action of progesterone in the new-born mouse. The report that progesterone given during the last third of gestation will cause fetal death has been confirmed, and it is suggested that this is due to a direct toxic effect of progesterone on the fetus. The rapid development of resistance to progesterone within a few days after birth suggests that a system or mechanism has appeared which is capable of detoxifying, catabolizing, counterbalancing or accelerating the excretion of progesterone.

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An Evaluation of the Role of Ferritin (VDM) in Traumatic Shock.* (19472)

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Introduction. In studies of shock, a correlation has been noted between irreversibility of the syndrome and a phase of hyporeactivity of the finer vessels of the exteriorized omentum and mesentery(1,2). The latter has been attributed to a blood-borne vasodepressor material (VDM) from ischemic liver, etc., which has been identified as a form of ferritin(3-5). The inference has been made that a release of ferritin occurring during shock contributes to the syndrome by rendering vessels unreactive (3). Few other vascular sites, however, have been demonstrated to react to VDM the way the mesoappendix does. The present experiments were designed to test whether 1) increasing the concentration of ferritin in tissues or 2) removing it from the circulation affects the ability of rats to survive after shock-producing trauma.

Materials and methods. Ferritin was crys-

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† Markle Scholar in Medical Science.

TABLE I. Effect of Increasing Ferritin in Tissues (Exp. I) and Removing it from Circulation (Exp. II) on Ability of Rats to Withstand Trauma.

Exp.	Group	No. of rats	Injection	% mortality	Mean death time (hr) ± stand. error
I	A	80	—	70	2.03 ± .216
	B	68	3 mg Fe as Hb	77.94	1.87 ± .187
II	C	46	.5 ml normal rabbit serum	76.09	1.39 ± .173
	D	43	.5 ml rabbit anti-dog ferritin serum	79.07	1.44 ± .148

talized by the method of Granick(6). It was recrystalized 4 times and finally reprecipitated 4 times by 50% saturation with ammonium sulfate. Thorough dialysis was carried out following each precipitation. Antiserum was prepared by injection of alum-precipitated ferritin into rabbits, according to the method described by Mazur and Shorr(4). The amount of ferritin was determined by the specific precipitin reaction(7) and by the slide technic modified after Granick(6). Estimation of ferritin was done by analysis of iron in the ammonium sulfate precipitate of the supernatant formed by heating tissue hemogenate to 80°C. The Wong(8) method for iron was used. The hemoglobin solutions used were prepared from human cells and were supplied us by Sharp and Dohme. The Noble-Collip drum(9,10) was used to produce traumatic shock. Our drum was constructed of 1/4 inch lucite. The inside diameter was 16 inches and the depth 6.5 inches. Two 2 inch baffles placed opposite each other were inside the drum, projecting inward at about a 45° angle. The drum was rotated at 45 r.p.m.

Results. Effect of increased concentration of tissue ferritin upon the outcome of traumatic shock. The ferritin content of tissues may be raised by injection of hemoglobin(11). In an early experiment, 1 mg doses (10 ml of the solution) of elemental iron as hemoglobin were injected intraperitoneally into 4 male Wistar rats every other day for 3 doses. One week after the final injection, the rats were sacrificed and their livers perfused with saline. The livers were then homogenized with twice their weight of distilled water and the homogenate heated to 80°C. The precipitate which formed was washed twice with water, which was pooled with the original supernatant. This supernatant was treated with 35 g of ammonium sulfate per 100 ml. The precipitate which formed was washed with 35% ammonium sulfate and finally analyzed for iron. Four controls were treated in the same manner. The control livers showed concentrations of iron in the crude ferritin fraction of 0.047-0.059 mg/g of liver. The iron concentration in these fractions from injected rats ranged from 0.072-0.108 mg/g. Additional analyses, done by the slide technic of estimation of ferritin crystals produced by cadmium sulfate, also indicated a significant increase in apoferritin in the rats receiving 3 mg of hemoglobin iron. The iron analyses showed the mean value of the injected group to be 69% higher than control. Results of the slide technic were 0 for controls and 3+ (2+ to 5+) for injected rats. Male Wistar rats of Carworth Farms' stock, weighing 150-200 g, were subjected to trauma in a Noble-Collip Drum one week after the injections of hemoglobin, employing the same dosage as above. Normal control rats were traumatized in the same manner. Each animal remained in the drum for 9 minutes, giving a total of 405 revolutions. Table I, Exp. I, gives the results of this experiment.

The significance of the difference between the mortalities of the two groups has been tested. The probability of 0.49 indicates that the difference between the two groups could have easily occurred by chance alone. To further analyze the possibility of a difference between the groups, the difference in the mean death times was tested. A probability of 0.28 again indicates the difference to be insignificant. Indeed, it is surprising that the large volume of hemoglobin injected (30 ml total) was not manifest as an undesirable insult.

Effect of decreased circulating ferritin upon the outcome of traumatic shock. An excess of antiserum of good titer to dog ferritin gives a recovery of about 99% of added rat ferritin (12). An intravenous injection of 0.5 ml of pooled anti-dog ferritin serum was given to 150 g rats immediately after drumming for 450 revolutions. The sera of these rats, sacrificed 30 minutes to 5 hours after drumming, retained readily detectable antibodies which precipitated rat ferritin. Those which survived the drumming showed antibodies in their sera which precipitated rat ferritin up to 24 hours.

To determine the effect of ferritin neutralization by antibody on the outcome of shock one group of rats was drummed for 450 revolutions and received 0.5 ml of the pooled anti-dog ferritin serum intravenously immediately upon removal from the drum. A similar group received 0.5 ml of normal rabbit serum after drumming and served as a control. Table I, Exp. II, shows the results of this experiment. The probabilities (0.83 and 0.73) calculated from these data indicate no significance in the differences in mortality or mean death time, respectively.

Since it has been our observation as well as Zweifach's(10) that animals dying within a few minutes after drumming or in the drum usually do so because of hemorrhage in the viscera or intracranially, only animals that survived at least 15 minutes are included in the tables. Mortality figures are based on a 24-hour survival time. However, the analyses of the data are unchanged when all animals are included.

Discussion. Chambers(1) pointed out that the omental circulation was selected for microscopic observation in the mesoappendix test because, in contrast to cutaneous tissue, its circulation is maintained throughout the greatest part of the hemorrhagic syndrome. Conclusions drawn from the reactivity of these vessels may in no way represent the reactivity of vessels generally in the organism. We feel that before a causal role in any phase of the shock syndrome can be assigned to ferritin (VDM), evidence other than its vasodepressor activity to certain vessel areas and its appearance correlated with the hyporeactive phase of shock must be obtained. Frank *et al.*(13) were unable to obtain correlation of VDM with irreversibility when morphine was used as the anesthetic. When unanesthetized animals were subjected to irreversible hemorrhagic shock, VDM failed to appear in the circulation. A correlation of blood borne VDM with the hyporeactive and irreversible phases of shock is suggested by them as an artefact produced by anesthesia. They offer evidence that the intravenous injection of VDM in hepatectomized, nephrectomized dogs had no effect on arterial pressure or survival. Similar experiments in this laboratory support their findings.

The presence of VDM in the circulation in shock and many other states(3), such as congestive heart failure, cirrhosis of the liver, etc., does not eliminate the possibility that it is the loss of some liver function which is responsible for the abnormal condition. In fact, our studies of ferritin storage in the liver suggest to us that after anaerobiasis and other liver injury ferritin would be found in the blood stream, especially in such small quantities as noted by bioassay. Our results indicate that the amount of ferritin in the tissues and its presence or absence in the blood are, however, not determinants of fatality due to trauma, nor is the time required for the animals to succumb to the insult significantly affected.

Summary. Increased tissue ferritin produced by intraperitoneal hemoglobin or precipitation of circulating ferritin by antiserum failed to affect mortality rate and mean death time in rats subjected to trauma in the Noble-Collip drum.

We are grateful to Drs. William P. Boger and Karl H. Beyer of Sharp and Dohme Co. for their efforts in supplying us with hemoglobin solutions.

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An Instrument Suitable for Measurement of Osmotic Pressure of Biological Fluids. (19473)

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In previous papers(1) I have reported on measurements of osmotic concentration of the extraocular and intraocular fluids. The instrument constructed for this special need has not been described and might be a valuable tool for similar investigations.

Niederl and Levy(2) published a simplified method for the determination of molar concentrations, a modification of the well known original method of Barger. The method is based upon the fact that in a closed system, harboring two solutions of different molarity,

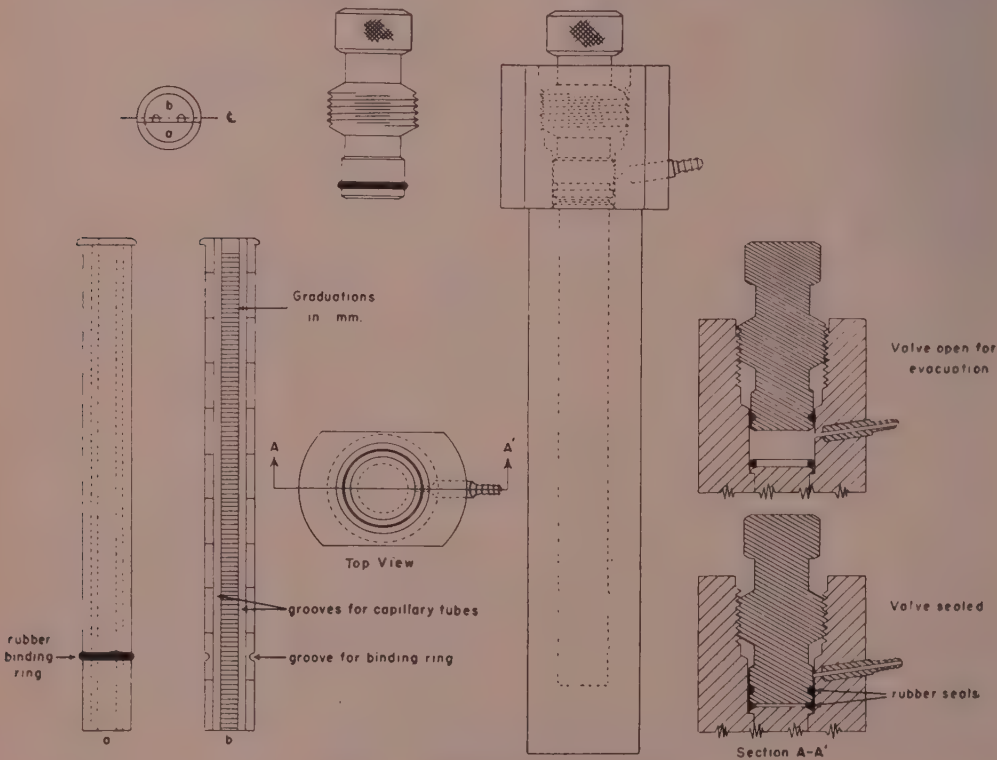

FIG. 1. Construction of instrument.

FIG. 2. Assembled instrument.

a change in the proportional volumes will occur due to a differential distribution of water vapor in the two solutions. The system tends towards an osmotic equilibrium, and the solution with higher molarity will gain, while the solution with lower molarity will lose in relative volume until equilibrium is reached. In this method 2 capillary tubes are filled with the relative solutions. On one end the capillaries are sealed and placed so that the menisci of the columns are exactly at the same level. The capillary tubes are now placed in a suitable external tubing which is sealed on one end, and the other is sealed only after evacuation of air to about 15 mm pressure. The system stays at room temperature for 4 days, when the distance between the 2 menisci, originally at the same level, is measured under low-powered microscopic magnification, using a micrometer scale in the eyepiece; the measurement is subsequently repeated every day for a week.

Applying the method for determination of osmotic pressure of biological fluids, we met with certain technical difficulties, one of which was the setting up of the capillary tubes in such a way that by handling or especially by centrifugation the menisci should not change their relative position. Exact arrangement of the two tubes was somewhat cumbersome. Another difficulty arose from the fact that biological material within such long periods of time as 4 to 10 days at room temperature, undergoes chemical changes in which larger molecules are broken down to smaller particles, disturbing the final result considerably. For this reason an improvement of the method of Niederl and Levy was attempted that would facilitate its application, and the time period necessary to obtain equilibrium in the system was considerably shortened. This last requirement for our purposes was fulfilled by reducing the volume of the evacuated space in which the evaporation and subsequent establishment of osmotic equilibrium take place. It can be assumed that the rate of exchange with time, t, of the amount of fluid evaporated, m, is given by a constant, α, minus a quantity which is proportional to the vapor pressure. This pressure is inversely proportional to the volume v. This gives a differential equation

$$\frac{dm}{dt} = \alpha - \frac{\gamma}{v} m \qquad (1)$$

the integral solution of which is:

$$m = \frac{\alpha v}{\gamma}\left(1 - e^{-\frac{\gamma}{v}t}\right) \qquad (2)$$

Therefore, if all other conditions are unchanged, reduction of the volume reduces the time in which equilibrium is reached. On the other hand, because the total amount of fluid evaporated is also reduced, the gain in time is somewhat biased by loss of sensitivity. If the reduction of volume is not excessive, this handicap is not considerable. In applying the technic of Niederl and Levy, in our hands the volume consisted of 5 or more cc, which in our modification could be reduced to 0.25 to 0.5

cc or any size desirable. This meant a reduction of the reading time by at least 10 times the original method. Leaving the system for 12 hours, *e.g.* overnight, yielded a satisfactory reading. No complete equilibrium was aimed at. In applying low microscopic magnification, estimation of differences less than 0.01 molarities can be obtained.

The instrument is constructed of lucite plastic. It can be used repeatedly for successive determinations. The transparency of the tubes allows the determination of the length of the fluid columns in the capillaries with the aid of low power magnification. The inner tube harboring the 2 capillaries possesses a recording grid. (The instrument may be obtained from Mr. V. L. Frykman at the Precision Instrument Shop of the University of Southern California in Los Angeles.)

Summary. The present paper describes an instrument constructed for determination of osmotic concentration in biological fluids. It estimates differences less than 0.1 molarities.*

* I take the opportunity to acknowledge the contribution of Dr. J. P. Meehn (Physiological Institute of the University of Southern California) in the design of this instrument.

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Potentiating Effect of Ascorbic Acid on Cortisone-Induced Gluconeogenesis. (19474)

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Previous work from this laboratory suggested that ascorbic acid treatment of rats, mice, and guinea pigs can prevent activation of the pituitary-adrenal axis in response to stressors(1-4). It was observed that the vitamin fails to alter the leukocyte response to cortisone and to adrenocorticotrophin. Furthermore, it was demonstrated that the vitamin does not possess any specific antiepinephrin action to explain failure of the activation of the pituitary-adrenal axis(4). Those data suggested that the ascorbic acid prevents the activation of the axis by either directly or indirectly rendering the pituitary inert to certain stimuli. Preliminary data indicated that ascorbic acid prolongs the leukocytic effects, and intensifies the gluconeogenic actions, of cortisone. The present communication reports data which indicate that the gluconeogenic action of cortisone is potentiated by ascorbic acid.

Materials and methods. These experiments were conducted on young adult male Swiss mice. The animals were bilaterally adrenalectomized under ether or nembutal anesthesia at least 3 days, and not more than 3 weeks prior to experimentation and maintained on an *ad libitum* fare of Purina Laboratory Chow and 0.9% NaCl solution. At the end of the periods of experimentation the animals were killed by a blow on the head, and the liver extirpated and plunged into a 30% solution of potassium hydroxide; the glycogen was then hydrolyzed by the method of Somogyi *et al.*(6), and the glucose determined by the method of Folin(5).

Experimental designs and results. The first experiment was conducted on male Swiss mice which had been adrenalectomized 3 weeks previously, and were maintained on Chow and saline. Body weight range at time of experiment was 25-30 g. Group I mice received single intraperitoneal injections of ascorbic acid (sodium ascorbate, vit. C, injectable, Roche), 100 mg per 100 g body weight, on the previous evening. Early the next morning these animals received similar amounts of the vitamin. The first injection

TABLE I. Liver Glycogen Concentration in Adrenalectomized Mice Treated with Cortisone and Ascorbic Acid, and with Cortisone and Saline

Group	No. mice	Treatment	Liver glycogen, mg/1 g liver ± S.E.	P
I	4	A,* C	49.1 ± 3.1	I vs II <.05
II	”	S, C	37.3 ± 2.1	II vs III <.01
III	”	0	20.6 ± 1.75	I vs III <.01

* A = Ascorbic acid; C = Cortisone; S = Saline.

TABLE II. Liver Glycogen Content in Adrenalectomized Mice Maintained on Glucose and Treated with Cortisone and Ascorbic Acid, or with Cortisone and Saline.

Group	No. mice	Treatment	Liver glycogen, mg /10 g body wt±S.E.	P
I	7	A,* C, G	29 ± 2.7	I vs II <.01
II	7	S, C, G	17 ± 2.4	II vs III <.01
III	5	G only	3 ± 1.2	I vs III <.01

* A = Ascorbic acid; C = Cortisone; S = Saline; G = Glucose.

was made in order to prime the animals. Immediately following the second injection of ascorbic acid the mice received single subcutaneous injections of cortisone (Cortone, Merck), 4.0 mg per 100 g body weight. Group II mice received similar treatment as those of Group I except that in this case vit. C (ascorbic acid) was replaced by intraperitoneal injections of physiological saline. Group III mice received no treatment. The animals were all taken off food following the injections, but were permitted to drink 0.9% NaCl solution. All animals were killed at the end of 4 hours after the injection of cortisone, the controls at the corresponding time, and glycogen determinations conducted.

The data are presented in Table I in which the liver glycogen concentration is presented as milligrams of glucose per 1 g of liver. It is seen that the liver glycogen content of the animals receiving cortisone is higher than that in the animals receiving no treatment. The liver glycogen content in the animals which received cortisone and vit. C (Group I) is significantly greater than that of the animals which received cortisone and saline (Group II) (P <0.05).

The second experiment was conducted on mice weighing 20 to 25 g. The procedure suggested by Venning *et al.*(7) for assay of corticoids was adopted in this experiment. Group I mice (adrenalectomized) received the previous evening the priming dose of ascorbic acid as those in the previous experiment, and were allowed to feed *ad libitum* overnight. The following morning these animals received 7 injections of a menstruum containing cortisone and glucose subcutaneously, the total dose of cortisone amounting to 70 μg, of glucose, 70 mg. These animals also received 2 injections of ascorbic acid, one at the time of the first cortisone injection, and the other at the time of the third cortisone-glucose injection, the amount of ascorbic acid being 100 mg/100 g body weight. Group II animals were treated like those of Group I, except that they received saline solution in place of the intraperitoneal injections of ascorbic acid. The animals of Group III received 7 injections of glucose, amounting to 70 mg, but received no cortisone, or ascorbic acid. The 7 injections mentioned above were given in the course of 6 hours, the animals being killed one hour after the last injection, *i.e.*, a total period of 7 hours after the first injection of the menstruum.

The data are presented in Table II in which the liver glycogen, as glucose, is presented as milligrams per 10 g of body weight of the animals. This is the method recommended by Venning *et al.*(7). It is observed that the liver glycogen content of Group II animals is 466% greater than that of the animals which received no cortisone (Group III); the livers of Group I mice contain 866% more glycogen than those of Group III animals. The liver glycogen content in the vitamin-cortisone mice is significantly greater (P <0.01) than that of the cortisone-saline animals.

Discussion. The data obtained from the 2 experiments indicate that ascorbic acid treatment of adrenalectomized mice supplied with small and large doses of cortisone increases the deposition of glycogen in the liver. This deposition of glycogen in the livers of the cortisone treated animals was probably due to gluconeogenesis. It is unlikely that the glucose supplied to the mice in the second experiment was converted to glycogen stores in

the liver; it is observed that the glycogen values in the animals receiving glucose alone were extremely low. Venning(7) had shown that in such glucose-treated animals, the glycogen deposition following cortical hormones was due to gluconeogenesis. The ability of ascorbic acid to enhance the liver glycogen deposition must therefore be ascribed to an increase in the gluconeogenesis due to the ascorbic acid in association with the cortisone, since the vitamin alone fails to enhance gluconeogenesis in adrenalectomized mice (unpublished data). McKee *et al.*(8) reported that liver glycogen deposition following the injection of cortical hormones into scorbutic guinea pigs was lower than that in normal animals. This observation could conceivably have been due to the general debility in the scorbutic animals. In the present study it is observed that vitamin treatment to animals on otherwise good ascorbic acid balance (the mouse requires little or no ascorbic acid in the diet) is capable of enhancing the glycogen deposition. This finding may be related to the action of the vitamin in normal intact animals exposed to stressors, in which case the alarm reaction is prevented, and survival is increased (9). It was shown that this ability of the vitamin requires the presence of the adrenal cortex, or of circulating cortical hormones(10).

It is possible that the vitamin "synergizes" with the existent cortical hormones in intact animals, and in animals supplied with cortical hormones. Data supporting this postulate come from the observation that the vitamin is capable of prolonging the leukocyte effects of cortisone(11). This prolongation, in addition to the demonstrated potentiation, of the action of cortisone by ascorbic acid suggests a synergism of ascorbic acid with cortisone. This synergism may explain the non-activation of the pituitary-adrenal axis in ascorbic acid-treated intact animals exposed to stress. It is possible that through this synergism the titer of cortical hormones is maintained in the blood, hence increasing the threshold of the anterior pituitary, according to the mechanism proposed by Sayers and Sayers(12). The relation of this possible synergism to normal adrenal secretion is being investigated.

Summary. The action of ascorbic acid treatment on the gluconeogenic action of cortisone was investigated in adrenalectomized mice. The deposition of glycogen in the liver is enhanced in animals treated with ascorbic acid in addition to cortisone. This observation is discussed in relation to the action of the vitamin in preventing the activation of the pituitary-adrenal axis in animals exposed to stressors. It is suggested that the vitamin may be synergistic with cortisone in some of its actions.

This study was aided by a Grant from the National Institutes of Health. The authors acknowledge the interest of Doctor Chester E. Leese. The ascorbic acid (Vit. C, Injectable, Na ascorbate, Roche) was supplied through the generosity of Doctor Roger A. Lewis, Hoffmann-La Roche, Nutley, N. J. Doctor Elmer Alpert, Merck and Co., Rahway, N. J., kindly supplied the cortisone (Cortone, Merck).

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Effect of Cortisone on Mast Cell Tumors (Mastocytoma) of the Dog. (19475)

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Included among the many effects of the adrenal cortical hormones on the organism is their ability to produce a marked reduction in the number of normal tissue mast cells. This has been observed with the use of ACTH in man(1) and with the use of cortisone in rats(2). Spontaneous tumors composed of neoplastic mast cells are of frequent occurrence in the cutaneous tissues of the dog(3). These growths may be benign or malignant, solitary or multiple, and range in size from nodules several centimeters in diameter to those weighing 300 or more g. They may be of unicentric or multicentric origin, and metastases are not uncommon in the regional lymph nodes, spleen, and liver in the malignant varieties. The microscopic structure consists of atypical histogenous mast cells whose cytoplasm contains metachromatic basophilic granules. The cells exhibit all gradations of cellular maturity and immaturity with fine and coarse granules which often can be correlated with the degree of malignancy of the various neoplasms. In the skin tumors the mast cells form nodular or diffuse sheet-like collections that heavily infiltrate the corium and subcutaneous tissue.

In view of the known effect of cortisone in causing a conspicuous reduction in the number of normal tissue mast cells, this agent was employed in the treatment of a dog with malignant multiple mast cell tumors.

Material and methods. The animal was an 8-year-old male white Spitz dog brought to my animal hospital for treatment. Three subepithelial tumors, 2 in the left shoulder region and one in the right upper lip, had been observed for a period of several weeks. The nodules ranged from 1.3 to 2.2 cm in diameter and were elevated from 4 to 6 mm above the surrounding skin. Under local anesthesia the nodules were surgically excised on 9/5/51. Examination of the animal on 12/1/51 revealed recurrence as manifested by the presence of 8 skin nodules in the left shoulder region and 7 skin nodules on the neck and back. These were similar in size and appearance to those previously removed. On 1/2/52 50 mg of cortisone (Cortone, Merck)* was administered intramuscularly twice daily and continued at this dose rate until 1/11/52. A nodule was excised on 1/5/52 and the remains of a nodule was excised on 1/11/52. Following each surgical procedure, the tissue was fixed in 10% formaldehyde solution and sections were stained with hematoxylin and eosin and Dominici's stain(4). Imprints were also made and stained with Wright-Giemsa stain.

Observations and results. The original skin tumors excised on 9/5/51 were a uniform pale tan color on section and not encapsulated. Microscopically the entire subepithelial tissue was completely replaced with dense diffuse masses of neoplastic mast cells and scattered eosinophils and lymphocytes. In sections the tumor cells exhibited moderate anaplasia and occasional mitotic figures were noted. The toluidine blue component of Dominici's stain revealed that the cellular cytoplasm contained relatively fine uniform sized metachromatic granules in ample numbers. The granules did not stain with hematoxylin as is not uncommon in more mature tumors with coarse granules. In the imprint preparations the fine discrete nature of the granules was more readily discernible and the cellular cytoplasm was seen to be intact and free from vacuolar and other degenerative changes (Fig. 1).

The gross appearance of all 15 recurrent nodules as observed on 1/5/52 after 3 days treatment with cortisone was identical with the same nodules before treatment except for a reduction of about 2/3 the pretreatment dimensions. This change occurred rather suddenly and was first noticeable on 1/5/52. Microscopic examination of sections of the excised nodule disclosed ample numbers of

* Dr. L. A. Michaud of Merck and Co. kindly supplied the cortisone.

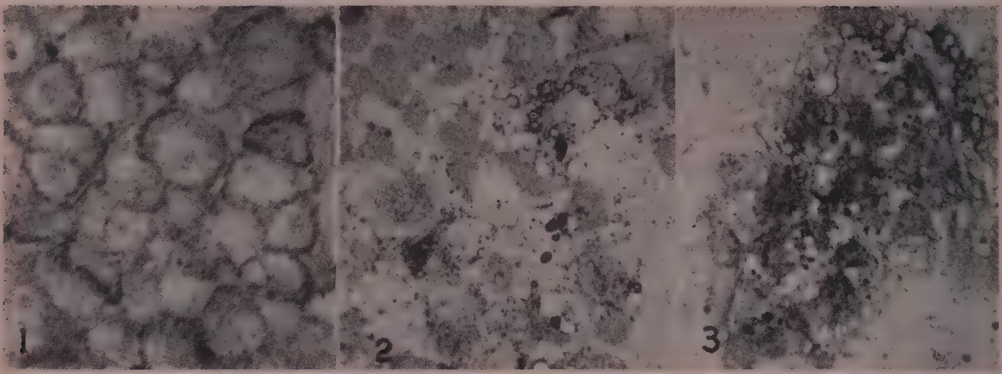

FIG. 1. Imprint of tumor mast cells prior to cortisone treatment showing intact cells with fine granules. Wright-Giemsa. × 600.

FIG. 2. Imprint of tumor mast cells after dog had received 300 mg of cortisone. The cells show vacuolization, granule clumping, granule scattering and disruption of the cellular membrane. Wright-Giemsa. × 600.

FIG. 3. Imprint of tumor mast cells after dog had received 900 mg of cortisone. Advanced degenerative changes, manifested by marked vacuolization, conglomeration of granules, disappearance of many fine granules and loss of cellular structure, are apparent. Wright-Giemsa. × 600.

mast cells with decrease in the number of eosinophils. Although many cells in the Dominici stained slides showed no alteration in the number, shape, size, and tinctorial characteristics of the granules, a greater number of cells exhibited specific cytoplasmic and granule aberrations. In these altered cells the cytoplasm sometimes contained small and large vacuoles, and the granules were frequently conglomerated to form irregular clumps several or more times larger than the original granules. The conglomerated masses often stained a deep purple-blue in contrast to the reddish-purple coloration of the fine granules. These cytoplasmic changes were especially conspicuous in the imprint preparations, and all gradations between intact tumor cells without degenerative alterations to those showing vacuolization, granule clumping, granule scattering and fraying of the cytoplasmic border were readily apparent (Fig. 2). In both imprints and sections the nuclei were well preserved.

From 1/5 to 1/11/52 the recurrent 14 nodules (one had been excised on 1/5/52) rapidly decreased in size so that by the latter date they had completely disappeared. All that remained was a pale tan coloration of the skin over the previous site of the nodules, which contrasted with the pale pink color of the surrounding normal skin. Microscopic examination of sections of the excised tissue revealed that the mast cells had almost completely disappeared. Practically no cells of normal structure were noted, and those present exhibited various degrees of degenerative change. The cells showed a ballooning of the cytoplasm caused by extensive vacuole formation. The intervacuolar cytoplasm consisted of an irregular fine tracery of sparse numbers of minute and conglomerated aberrant staining metachromatic granules that indistinctly outlined and occasionally occurred within the vacuoles. The cytoplasmic borders were indistinct and frayed. Very few nuclei appeared normal; many had disappeared and others were pyknotic. The eosinophils were also fewer in numbers. The lymphocytes, on the other hand, appeared to be increased; however, this increase was relative inasmuch as the other cellular elements were numerically reduced. The degree of degenerative alterations of the mast cells was particularly noticeable in the imprints (Fig. 3). In these, solitary or groups of mast cells were hardly recognizable as such. Instead, the cytoplasm consisted of a mass of vacuoles in and between which were irregularly distributed varying sized conglomerated granules with few of fine structure. The cellular borders were indis-

tinct, frayed and hazy. The nuclei were likewise difficult to outline, although they exhibited a better state of preservation than the cytoplasm.

Examination of the animal on 2/21/52 indicated no recurrence of the tumor nodules that had disappeared following cortisone therapy. However, a new nodule 1½ by 0.6 cm had appeared in the right lower lip and the regional lymph nodes were enlarged. Up to the present day, no additional nodules or recurrence of the previous nodules has appeared.

During the course of cortisone therapy the animal showed no unusual symptoms and appeared to be normal. No abnormal bleeding tendency occurred during or after surgical excision of the nodules.

Discussion. Of especial interest is the complete macroscopic regression of the mast cell tumors following cortisone treatment. This observation was confirmed microscopically inasmuch as the mast cells had almost completely disappeared and that the relatively few remaining cells were all in various stages of degeneration. Such involution has never been noted to occur spontaneously in personal observation of over 100 canine mast cell tumors; instead, the usual sequence in malignant types is a progressive intensification of the neoplastic process with terminal death. Although it would be premature to suggest that the disappearance of the tumors is permanent, 61 days have thus far elapsed up to the present writing without recurrence. The new nodule, presumably a mast cell tumor, that appeared after cessation of cortisone indicates that the neoplastic process has not been arrested. Further observations are being conducted and if additional tumors occur further cortisone treatment will be instituted.

The sequence of changes noted in the tumor mast cells are similar to those seen in normal mast cells following cortisone therapy. The cytoplasmic vacuolization, conglomeration and altered staining properties of the metachromatic granules, disappearance of many fine granules, fraying and disruption of the cellular membrane, cellular destruction and granule scattering seen in sections and imprints are essentially the same as that observed in aging tumor mast cells cultivated *in vitro*(5). The degenerative changes of the tumor mast cells that eventually lead to their death, whether due to aging or hormonal substances, are thus alike in type and degree. Duplications of these cellular alterations have also been noted in normal tissue mast cells *in situ* without cortisone therapy, but have usually been thought of as artifacts(6).

Since it has been determined(7) that the canine mast cell tumor may contain almost 50 times as much heparin as has been obtained from the richest normal source of the most active form of heparin, one might expect that the treated animal should exhibit interference with the coagulation of its blood. Although the circulating heparin-like substances, which rapidly rise in man after the administration of cortisone(8,9), were not determined, no unusual bleeding tendency existed as the surgical procedures were not accompanied by excessive hemorrhage. The explanation for the normal clotting time in the treated animal might be that insufficient amounts of heparin were liberated to have an anticoagulant effect or that inactivation or destruction of heparin followed its release from the cells. This anomaly, the presence of large numbers of mast cells and a normal state of blood coagulation, is not peculiar to these experiments where the animal was treated with cortisone; similar normal clotting time of the blood has been repeatedly observed during surgical manipulation and removal of mast cell tumors in dogs who have not received cortisone.

Although the therapeutic effectiveness of cortisone in all malignant mast cell tumors may be speculative, the results obtained in the cited animal are encouraging and warrant further investigation.

Summary. The effect of cortisone was studied in a dog with malignant multiple mast cell tumors (mastocytoma). A total of 900 mg was given intramuscularly at the rate of 100 mg daily. The tumors rapidly regressed in size so that by the 9th day of treatment they had completely disappeared macroscopically. Microscopically the neoplastic mast cells showed cytoplasmic vacuolization, conglomeration and altered staining reaction of the metachromatic basophilic granules, disap-

pearance of many fine granules, fraying and disruption of the cellular membrane, granule scattering, and, finally, cellular destruction and disappearance. The encouraging results obtained in the treated animal warrant further investigation of cortisone in the treatment of dogs with malignant mast cell tumors.

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Urinary Excretion of Hydrazine Derivatives of Isonicotinic Acid in Normal Humans.* (19476)

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Recent reports have shown that isonicotinyl hydrazide (Rimifon) and 1-isonicotinyl-2-isopropyl hydrazine (Marsilid) are effective antitubercular agents in mice(1,2), guinea pigs and rabbits(3), monkeys(4), and humans(5-8). We have described an assay method and determined levels of Rimifon and Marsilid in blood plasma after oral dosage to humans, dogs and mice(9). In this method, the hydrazides are converted by permanganate treatment to isonicotinic acid, which is determined colorimetrically by the reaction with 10% cyanogen bromide and ammonia used by Mueller and Fox(10) for nicotinic acid assay. Preformed isonicotinic acid is determined by assay *before* permanganate oxidation. In the experiments described below, the cyanogen bromide-ammonia reaction has been applied to untreated and permanganate-treated urines to study the products and rate of excretion by normal humans of oral doses of Rimifon and Marsilid.

Experimental. Nine normal subjects (laboratory personnel), 2 females and 7 males ranging in age from 26 to 43 and in weight from 56 to 82 kg, participated in the urine excretion tests. To determine the color produced by normal urines in the cyanogen bromide reaction, pre-dosage control urines were collected on each of 2 successive days. Collections were started at 9 A.M. and made at intervals ending after 1, 2, 3, 8 and 24 hours. On the test days, a single oral dose of Rimifon or Marsilid was given at 9 A.M. and urine collections made at the same intervals for 24 hours. A pooled sample was collected by each subject for the second 24 hours after dose. Diets were not controlled, but the subjects were instructed to follow as nearly as possible the same diet on each of the collection days. In the first test, the subjects were divided into 2 groups and given 250 mg of Marsilid or 125 mg of Rimifon. These doses were reversed in all subjects in the second test; in the third test, all subjects received 192 mg of Rimifon, which is equivalent on a molar basis to 250 mg of Marsilid. At least one week was allowed between successive doses. Both control and test urines were assayed directly and also after permanganate treatment, using the reaction with 10% cyanogen bromide in ammonia buffer as previously described(9). For direct assay, 1 ml of urine plus 5 ml of buffered ammonia reagent were

* Roche Publication No. 306.

TABLE I. Relative Magnitude of Blanks in Control Urines and Isonicotinic Acid Levels in Test Urines. (All values are expressed as mg of "isonicotinic acid" and represent averages for 9 subjects).

	Direct colorimetry					Colorimetry after $KMnO_4$ treatment				
	Blank in control urines			Free isonicotinic acid in test urines		Blank in control urines			Total isonicotinic acid in test urines	
Hr	1st day	2nd day	Dev. from mean	After Rimifon*	After Marsilid*	1st day	2nd day	Dev. from mean	After Rimifon*	After Marsilid*
1	1.92	1.29	.32	3.3	2.9	1.66	1.23	.22	8.6	3.8
2	1.40	1.61	.11	5.5	5.9	1.15	1.65	.25	13.3	7.7
3	1.86	1.39	.24	4.5	6	1.68	1.55	.07	10	7.6
3–8	6.8	6.3	.25	15.3	19.4	6.9	5.7	.6	36.7	26.2
8–24	13.4	17.3	2	18.8	35.2	12.9	13.5	.3	34.3	47.7
Total	25.4	27.9	1.3	47.4	69.4	24.3	23.6	.4	102.9	93
24–48	"	"	"	5.5	12.6	"	"	"	6.5	16.9

* Oral dose of Rimifon = 192 mg and of Marsilid = 250 mg; in each case the dose is equivalent to 172 mg of isonicotinic acid.

diluted to 25 ml of water. After mixing and centrifuging, a 2 ml aliquot was taken for colorimetry.

For the permanganate treatment (to split Rimifon or Marsilid to isonicotinic acid), 1 ml of urine in a 50 ml beaker was diluted with 10 ml of water. Four percent potassium permanganate was added dropwise until a definite pink color persisted and the solution boiled down slowly (in about 30 minutes) to a volume of about 5 ml. The addition of permanganate was repeated during the heating, if necessary to maintain a pink color. After heating, 1% ascorbic acid was added to decolorize and the solution transferred quantitatively to a 25 ml graduate. Five ml of buffered ammonia reagent were added before dilution to 25 ml with water. A part of this solution was centrifuged for 5 minutes at 2000 r.p.m. and a 2 ml aliquot of the clear supernatant taken for colorimetry. The *galvanometer reading* for each urine sample was converted into mg of "isonicotinic acid" on the basis of isonicotinic acid standard readings, which were taken at frequent intervals. Subtraction of the average control value for a given sample from the corresponding test value gives the level of "isonicotinic acid" in the test urine. Pending isolation and positive identification of the substances measured by the above colorimetric tests, which are being investigated by ion exchange and chromatographic methods, the difference between test and control values is referred to as "free isonicotinic acid" for the direct assay and as "total isonicotinic acid" (*i.e.* free isonicotinic acid plus the amount formed on splitting Rimifon and Marsilid) for the assay after permanganate treatment. The increase in color upon treatment is taken therefore as a measure of Rimifon or Marsilid excretion, although it must be pointed out that the product measured may be a conjugated form of the drug or a related substance which behaves similarly in the cyanogen bromide reaction, *i.e.* one which gives practically no color until treated with permanganate. In calculating free isonicotinic acid levels, a correction has been made in each case for the slight color obtained from the Rimifon or Marsilid *per se*(9).

The validity of the estimation of isonicotinic acid in test urines is dependent upon the day-to-day reproducibility of the blank color in normal urines. Table I shows the average blank values for the two control days and gives a comparison of deviations from the mean blank with increases found after ingestion of Rimifon or Marsilid. Although the blank values are high, the average day-to-day variations are small in relation to the increases found in the test urines. Hence, the average test excretions shown are subject to a relatively small error due to blank variations in direct colorimetry and are practically unaffected by these variations after permanganate treatment.

Results. The excretion patterns are shown graphically in Fig. 1, 2 and 3 for doses of

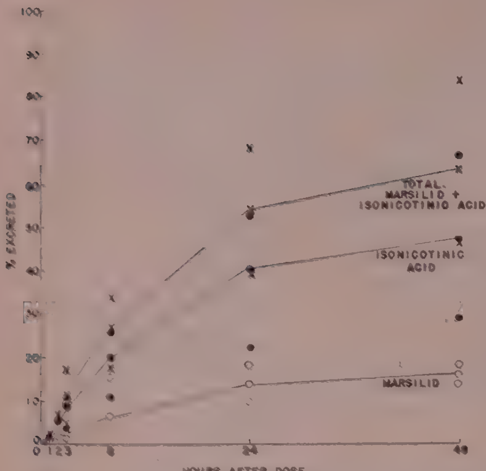

Fig 1

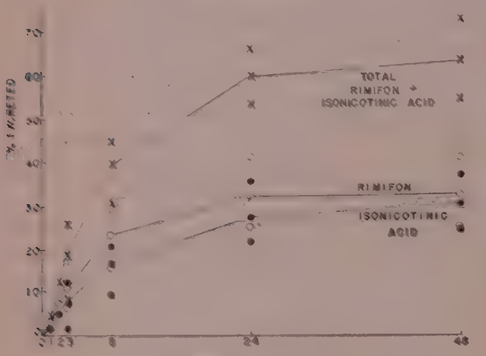

Fig 2

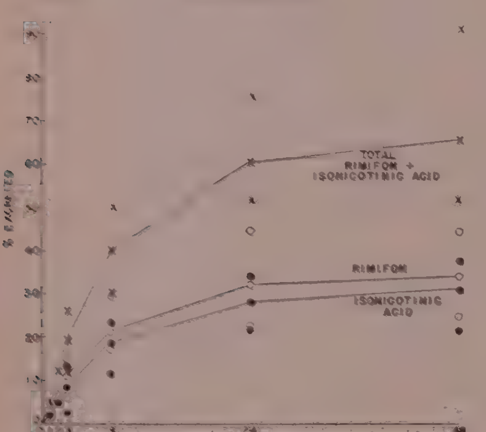

Fig 3

FIG. 1. Average and range of human urinary excretions by 9 normal subjects after an oral dose of 250 mg of Marsilid.

FIG. 2. Average and range of human urinary excretions by 9 normal subjects after oral dosage of 192 mg of Rimifon.

FIG. 3. Average and range of human urinary excretions by 9 normal subjects after an oral dose of 125 mg of Rimifon.

250 mg of Marsilid and 192 and 125 mg of Rimifon, respectively. The lines represent average excretions and the individual points on either side the corresponding minimum and maximum levels for the 9 subjects. The average percentage excretion of Rimifon is greater than of Marsilid, the ratio being higher than 4:1 in the first 3 hours after dose and ranging down to about 2:1 for the total 48-hour excretion. Since the percentage excretion of free isonicotinic acid is practically the same in the first 8 hours after both Rimifon and Marsilid, the total absolute excretion after Rimifon is greater in this period. On the basis of the greater retention of Marsilid, higher blood levels of the latter might be expected. Preliminary assays of blood plasma in this laboratory confirm the fact that levels of Marsilid are higher than of Rimifon after equimolar dosage in humans(11). Similarly, in dog plasma studies(9), in which equal oral doses on a weight basis (*i.e.* a smaller dose of Marsilid on a molar basis) were given, Marsilid showed higher maximum values as well as higher levels after 4 and 8 hours. On a 24- or 48-hour basis, a higher percentage of Marsilid is split and excreted in the urine in the form of isonicotinic acid; this difference between Rimifon and Marsilid may be due to the longer retention of the latter in the body, which allows more time for the splitting to occur. After 48 hours, the total percentage excretion is practically the same after both compounds. The excretion patterns at the two dose levels of Rimifon (Fig. 2 and 3) are almost identical.

Discussion. Although the reaction with 10% cyanogen bromide in ammonia buffer is not specific for isonicotinic acid, it has the advantage of providing a means of differentiating the latter from either Rimifon or Marsilid, which give only a very small percentage of the color obtained with an equivalent amount of isonicotinic acid. An adequate correction for non-specific color in normal urines has been obtained by assaying parallel control samples before dosage. The metabolite responsible for the increased color on direct

assay after dosage has been referred to as isonicotinic acid, but the latter has not yet been isolated and identified. The substance measured is obviously not the unchanged hydrazide since Rimifon or Marsilid *per se* could account for only a small fraction of this color. It is also not niacin or niacinamide since the reaction with 3% cyanogen bromide and Metol, which measures the vitamin or its amide but not isonicotinic acid(9), did not yield significantly greater color with the test urines than with the controls. It is also not an N^1-methyl derivative of isonicotinic acid or its hydrazide since the methiodide of isonicotinic acid hydrazide (Ro 2-5027) gave no color either before or after permanganate treatment. Another possible metabolite is isonicotinamide, but the latter yields only about one-half the color given by isonicotinic acid in the 10% cyanogen bromide-ammonia reaction. Since the 48-hour excretion calculated as isonicotinic acid ranged as high as 67% of the dose, the color can obviously not be due entirely to isonicotinamide. On the basis of the above evidence, it appears likely that the direct assay measures either isonicotinic acid or a mixture of the latter and isonicotinamide. The possibility exists, however, that there may be present other closely related derivatives which are capable of reacting similarly with 10% cyanogen bromide and ammonia. The identity of the component in the test urines responsible for the increased color after permanganate treatment has not been established, but this behavior is typical of both Rimifon and Marsilid. Isonicotinamide shows no such increase in color. Pending further investigation, the colorimetric response has been calculated as Rimifon or Marsilid. It is possible that the substances measured may be conjugated or otherwise modified forms of the hydrazides. If the compound determined after permanganate treatment is isonicotinic acid, then the total percentage excretions shown in Fig. 1, 2 and 3 will remain unchanged regardless of the nature of the excretion products.

It is noteworthy that Marsilid is excreted at a much lower rate than Rimifon in the first 8 hours after dose. The effect of this higher retention of Marsilid on the relative blood levels of both substances is being investigated and preliminary evidence indicates that plasma levels of Marsilid in tubercular patients reach higher peaks and are of longer duration than the levels of Rimifon after equimolar oral doses(11). The significance of this difference in relation to the anti-tubercular activities of the two compounds remains to be evaluated.

Summary. Urinary excretions after oral dosage of Rimifon and Marsilid to 9 normal humans have been studied colorimetrically by means of the reaction with 10% cyanogen bromide in ammonia buffer. The influence of non-specific chromogens has been eliminated to a large degree by parallel assays of predosage control urines. The concentration of metabolite in which the hydrazide linkage has been split was determined by direct assay and the results expressed as "free isonicotinic acid" pending positive identification. The increase in colorimetric readings upon permanganate treatment has been ascribed to unchanged Rimifon or Marsilid, although, again, the exact nature of these excretion products has not been ascertained. Total excretions in 48 hours average about 65% of the dose of both Rimifon and Marsilid, but in the same period the excretion of unhydrolyzed Marsilid is only about half that of Rimifon. In the first few hours after dose, the relative rate of excretion of Marsilid is still lower, averaging only about one-fourth that of Rimifon. Approximately half of the Rimifon is split prior to excretion and almost identical excretion patterns are found at the 192 and 125 mg dose levels. In case of Marsilid, the excretion of free isonicotinic acid parallels closely its excretion after Rimifon for 8 hours, but the 24- and 48-hour totals of free isonicotinic acid are about 50% higher after Marsilid. This increased excretion of the split product results apparently from the longer retention of Marsilid in the body.

Addendum. While the present manuscript was in press, a paper by Elmendorf *et al.*(12) appeared in which 24-hour, urinary excretions by 6 patients of isonicotinyl hydrazide after a single oral dose of 3.0 mg per kg (total dose = 140-200 mg) are reported to range from 47.8 to 70.7% of the dose (average =

61.0%). These excretion values, determined colorimetrically by the p-dimethylaminobenzaldehyde reaction(13) are almost twice as high as those reported above for 9 normal subjects (average excretion of Rimifon after 125 mg dose = 32% and after 192 mg dose = 33%) and correspond closely to the total excretion of isonicotinic acid plus Rimifon as determined by the cyanogen bromide-ammonia method (average total excretion of both 125 and 192 mg doses = 60%). This difference may be due to a) non-specificity of the p-dimethylaminobenzaldehyde reaction or b) a difference between tubercular and normal subjects. Although no Rimifon data have been obtained by the cyanogen bromide test on tubercular patients, the latter possibility appears unlikely in view of the similarity in excretion patterns of Marsilid by both types of subjects. Five male tubercular patients excreted approximately the same percentage of Marsilid in 24 hours as the above normal subjects (10-18%), the Marsilid in both groups being determined from the difference between assays by the cyanogen bromide method before and after permanganate treatment. Since isonicotinic acid, which is measured by direct assay with cyanogen bromide, does not react with p-dimethylaminobenzaldehyde, the differences in Rimifon excretions by the two assay methods can not be due to the isonicotinic acid present. It is possible that the hydrazine split from the Rimifon appears in the urine in another form which is measured by the p-dimethylaminobenzaldehyde reaction.

The Rimifon excretions by normal subjects as determined by the cyanogen bromide test have been confirmed microbiologically by the method of Tabenkin *et al.*(14), which measures Rimifon but not hydrazine or isonicotinic acid. Pooled, 24-hour urines from the test shown in Fig. 2 yielded microbiological assays for Rimifon, which were generally in close agreement with the chemical values.

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A Method of Positive Pressure Anesthesia for the Rat.* (19477)

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Herein described is a method of positive pressure anesthesia for the rat that has proved to be superior to those methods employing intubation and tracheotomy.

The apparatus consists of 2 Erlenmeyer flasks joined by rubber tubing to a positive pressure mask (Fig. 1). Flask A contains water and receives oxygen from a standard tank. The water serves to humidify the gas

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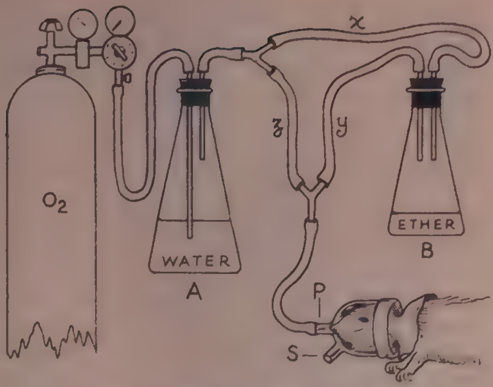

FIG. 1. Closed positive pressure system with mask attached. Letters x, y, and z indicate clamping points on tubing for the variation of the oxygen-ether mixture. The rat's head is shown in proper position.

and also acts as an indicator of its rate of flow. Flask B contains ether and is connected into the system in parallel with Flask A and the mask. The glass tubing used has an external diameter of 0.7 cm. The positive pressure mask consists of a rounded glass chamber of sufficient depth (4.5 cm) and diameter (4 cm) to receive the head of the animal (Fig. 1). The inlet tube (P) is connected to the oxygen-ether supply. A second tube (S), 2 cm in length and 1.4 cm in external diameter, leads directly to the outside. The opening of the mask is equipped with a rubber cuff that permits the entrance of the head and neck and forms a tight seal without constriction. This cuff is readily obtained by removing the thenar and thumb section from a size 7½ or 8 standard surgical glove. The tip of the narrower thumb section is removed. The remainder of the thumb section is then fitted over the rim of the mask. The resulting orifice can be varied as desired by increasing or decreasing the overlap of the thumb section upon the body of the chamber. The remaining rubber flange is tightly tucked about the animal's neck to produce a better seal. As the esophageal orifice of the rat is considerably larger than the laryngeal, the oxygen-ether mixture passes more easily into the stomach than into the lungs when it is delivered under positive pressure. To prevent this, counterpressure is exerted upon the abdomen by means of a tight abdominal binder that can easily be made by cutting a strip of rubber 4 centimeters wide from a surgical glove.

Method. The animal is first anesthetized with ether in a bell jar. Upon applying the mask, care must be taken that the rubber cuff is not applied too tightly about the neck. The flow of oxygen is controlled by the standard gauge attachment of the oxygen tank. A flow of 1 to 4 liters per minute is usually sufficient depending upon the size of the rat. The suitability of the flow-rate of the oxygen-ether mixture is easily judged by the degree of distention of the rubber portion of the mask. The concentration of ether and oxygen is varied as necessary by the application of a surgical clamp at appropriate positions on the rubber tubing in the system (Fig. 1, x, y, z). In general, a satisfactory level of anesthesia can be maintained by leaving the entire system open. The anesthetist controls the pressure within the mask, and the respiratory rate, by opening or closing the outlet tube (Fig. 1, S). The transparency of the glass mask permits constant observation of the color of the mucous membranes.

This method has been used successfully in over 130 thoracotomies. Eighty-seven of these were for ligation of the left pulmonary artery. With mastery of the technic there have been no operative deaths in the last 50 operations. This compares very favorably to the loss of 3 out of every 5 animals by Ellis and his associates(1) for the same operation using the translaryngeal intubation method described by Porter and Small(2).

The method described here has several advantages over that of Porter and Small(2) in that it eliminates the necessity of intubation, the danger of trauma to the upper respiratory tract, and the possibility of obstruction of the intratracheal tube. The use of ether alone permits not only close control of the anesthesia, but also a short post-operative reaction period. Neither of these is possible with the use of intraperitoneal barbiturates. Although rats are unusually prone to respiratory diseases, the number of post-operative deaths following the use of ether has been reasonably small.

The tracheotomy method of positive pressure anesthesia as described by Farris(3) has limited application. It was designed for use in terminal experiments where the survival of the animal is not necessary.

As an additional aid in the performance of the thoracotomy, a special operating table easily constructed from a standard cigar box is used. The extremities are tied in such a way that the animal is partly suspended and partly supported upon a soft-rubber sponge. This permits more freedom of thoracic movement and easier expansion of the unoperated lung. The use of the rat-board has proved to be more restricting and the results have not been as satisfactory.

Summary. A method of positive pressure anesthesia for the rat using a mask and an abdominal binder is described. It is applicable to the use of ether, intraperitoneal barbiturates or both as anesthetic agents. A closed system for the administration of the oxygen-ether mixture is also illustrated.

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Occurrence of a Sodium-Potassium Antagonism in Nerve Block.* (19478)

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It is recognized that sodium ions are required for activity of nerve fibers(1-6). It is also well known that potassium ions, in concentrations above normal, cause a depolarization of the nerve membrane, a decrease in excitability and a block of conduction(4, 7-9). A long series of experiments has culminated in the theory that entrance of sodium during the period of the rising phase of the action potential spike, and the exit of potassium during the falling phase, are central events in the mechanism of conduction(5). This theory recognizes the important point that the movements of these two ions are not independent and unrelated events but rather, integrated processes which occur in sequence. The sodium and potassium mechanisms thus interlock at some point in the chain of reactions of the nerve impulse. The problem, therefore, is to discover the means whereby such interlocking occurs. This communication will report an interaction between sodium and potassium in the maintenance of conduction in frog nerve fibers which seems to be of more than passing interest. At this stage of development it is only necessary to present a simple and factual report of the results without interpretation.

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Procedure. Except for certain essential information, no details of procedure need be given, since these were reported in a previous communication(6). Isolated and desheathed sciatic-peroneal nerves of bullfrogs were laid across stimulating and recording electrodes in a moist lucite box. A 20 mm segment of nerve between the stimulating and the recording electrodes was set within a glass cup. Various test solutions were added to the cup, thus exposing the segment to the solutions. Phosphate Ringer's solution at a pH of 7.0-7.3 was used. This contained 0.11 M NaCl and 0.0018 M KCl in addition to the other usual components of frog Ringer's solution. Potassium enriched solutions, when required, were made by adding crystalline KCl to unaltered Ringer's fluid. As Shanes(10) has pointed out, this procedure is not only permissible but is requisite in a system which is

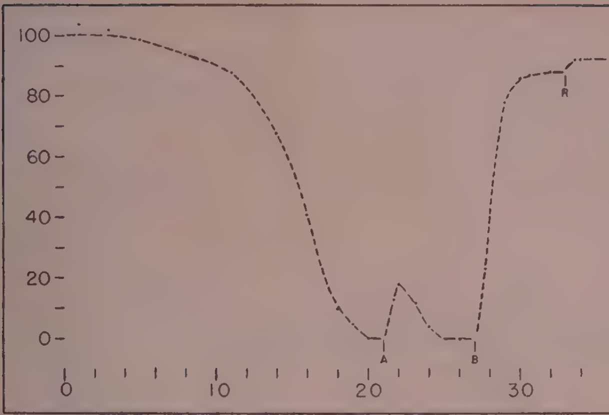

FIG. 1. The action of potassium ions on recovery of conduction at low, and at normal sodium ion concentrations. In all 3 figures the height of the compound A spike, in % of normal, is plotted against time, in min. At zero time the nerve segment was treated with .11 M tetramethyl ammonium bromide with no added NaCl. Block occurred, and at 21 min (A) a solution containing .0085 M NaCl and .0108 M KCl was added in place of the low sodium solution. At 27 min (B) a solution containing .11 M NaCl and .0108 M KCl replaced the previous solution. Ringer's solution with the normal concentration (.0018 M) of KCl was added at 33 min (R).

relatively impermeable to sodium and permeable to potassium and chloride. Sodium deficient solutions were prepared by osmotic replacement of the NaCl with compounds such as tetramethyl ammonium bromide or choline chloride. As already indicated(6) these compounds may be considered to be inert replacements for the NaCl. The significant experimental procedure involved the recording of the effect on conduction in the A fibers, of solutions with different potassium and sodium concentrations. Conduction in the A group of fibers was studied by oscillographic recording of the compound A spike in the nerve region beyond the treated segment.

The *results* are understandable by reference to the accompanying three figures and their legends. The nerve fibers were first blocked by a 0.11 M tetramethyl ammonium bromide solution containing no added NaCl. Except for Fig. 1, the time course of this low sodium block is not shown because this was described elsewhere(6). In addition to the block, Fig. 1 illustrates successively the effects of adding 2 solutions each with 6 times the normal concentration of KCl, the first solution (added at A) containing 0.0085 M NaCl, and the second solution (added at B) containing 0.11 M NaCl. This experiment brings out the point that a solution with potassium ions at 6 times the normal level was able to restore and to maintain conduction with the

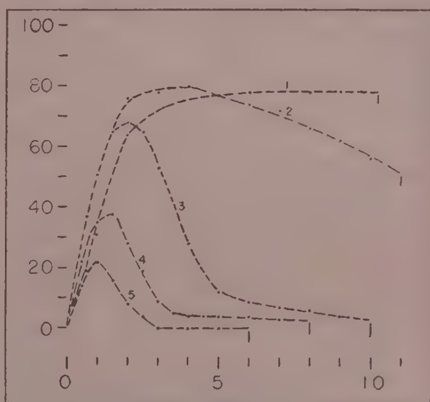

FIG. 2. Influence of potassium ions on recovery from low sodium block. Recovery was followed with solutions containing .0085 M NaCl and with varying concentrations of KCl. Each solution was added one min after completion of low sodium block. KCl concentrations were: .0036 M (1), .0054 M (2), .0072 M (3), .009 M (4) and .0108 M (5). To avoid complicating the figure the return of the A potential after the addition of Ringer's solution at the times indicated by vertical lines has not been drawn in.

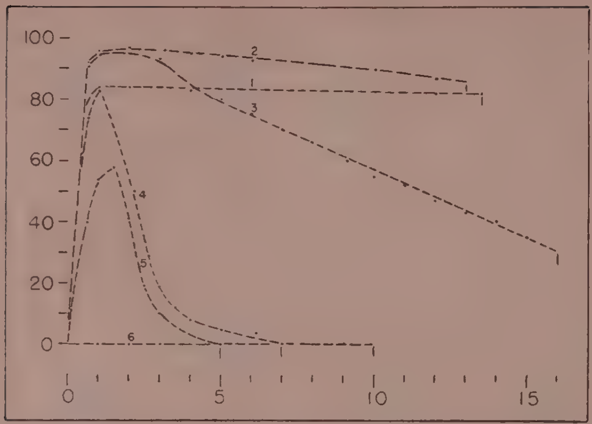

FIG. 3. Action of potassium ions in presence of varying concentrations of sodium. Recovery from low sodium block was followed with solutions containing .009 M. KCl in the presence of NaCl at the following concentrations: .11 M (1), .066 M (2), .044 M (3), .022 M (4), .011 M (5) and .0055 M (6). Not drawn is the recovery which followed the addition of Ringer's solution at the times indicated by the vertical lines.

higher sodium concentration but not with the lower sodium concentration. It is known already(6) that a solution with 0.0085 M NaCl in the presence of normal potassium is able to maintain activity in many of the A fibers. This finding is supported by the graphical data of Fig. 2.

The second figure shows the course of recovery from low sodium block by means of different solutions all containing 0.0085 M NaCl but with varying potassium chloride concentrations. It is clear that though the 0.0085 M concentration of NaCl was able to effect recovery and to maintain conduction in the presence of 0.0036 M KCl, it was less able to do so, the higher the potassium concentration. Fig. 3 illustrates the converse effect, that is the action of different solutions with varying sodium concentrations and with one level of potassium chloride, *i.e.*, 0.009 M. This experiment shows that a concentration of 0.11 M NaCl was sufficient to antagonize the action of 0.009 M KCl so that recovery occurred and no secondary block developed, at least within the period of the experiment. However, lower concentrations of NaCl were unable to maintain activity in the presence of 0.009 M KCl.

The results of these experiments reveal the existence of a marked antagonism between these physiologically significant ions. Relatively small changes in potassium concentration were able to modulate the action of sodium. In addition to the primary problem of the mechanism of this antagonism, these results raise the interesting possibility that the sodium-potassium antagonism may be the basis for a number of physiological properties of nerve activity which are as yet unexplained. The refractory period, for example, is one such property. If sodium action is required for spike production, and if potassium leaves the nerve fiber at an accelerated rate during the falling phase of the spike, then the momentary rise in external potassium in the neighborhood of the fiber after an impulse may lead to a momentary antagonism to sodium and be the cause of refractoriness.

Summary. The conduction block in bullfrog A fibers which is produced by the presence of elevated potassium ions is dependent on the sodium concentration. At a fixed level of potassium the rate of block increases as the sodium concentration is decreased. This occurs at concentrations of sodium which, at normal potassium, have no blocking action.

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Influence of Hemagglutinating Viruses on Tumor Cell Suspensions: I. Growth Inhibition and Reversal of the Effect.* (19479)

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In the course of experiments with mixtures of Newcastle Disease (NDV) virus and suspensions of sarcoma 180 cells it was found that after being kept at 4°C, the tumor cells failed to grow when inoculated subcutaneously into mice. This result was accompanied by a fall in hemagglutination titer of the virus. When similar mixtures were kept at 4°C for 10 minutes and subsequently at room temperature for 1, 2, or 3 hours, subcutaneous inoculation resulted in tumor growth. This was accompanied in most instances by the reappearance of hemagglutinating activity of the virus. The similarity of these findings to those observed when hemagglutinating viruses are brought in contact with red cells (1) has led to further experiments to determine to what extent the parallelism of the phenomenon holds. Most have been conducted with the NDV and the sarcoma 180 suspensions, but other tumor-virus systems have been used with success. The preliminary results are herewith reported.

Materials and methods. Viruses. The Massachusetts strain of NDV† was used in most of the experiments. In a few, however, the PR8† strain of influenza and the WS† strain of neuro-influenza were employed. All were used as pools of allantoic fluids. *Tumor suspensions.* Animals bearing 7-day-old sarcoma 180 were sacrificed, their tumors removed and made into a suspension by pushing them through a fine 40 mesh monel metal screen with a pestle. The suspensions were made to 10% in Locke-Ringer buffered with phosphate to make the pH 7.2. Glucose was added to make 600 mg %. The suspensions consisted of single cells, clumps of 8-10 cells and larger clumps up to the size of the apertures of the sieve. After the larger clumps had been permitted to settle a fairly uniform suspension was obtained. Both settled and unsettled suspensions were used in the experiment. Suspensions of Erhlich carcinoma were made in the same way.

Experimental procedure. In most experiments 10% sarcoma 180 and virus were mixed at the desired temperature. At different time intervals 0.5 cc of tumor-virus mixture was inoculated subcutaneously in each flank of Carworth Farm white mice. A readily palpable and visible bleb formed which gradually disappeared in about 1 hour. When tumor grew it assumed the form of the bleb. The mice were examined at weekly intervals and tracings of their tumors made. They were kept for 1 month after which they were autopsied to ascertain the presence of internal tumors. The results are expressed as per-

* This work was supported by funds from the National Cancer Institute of the U. S. Public Health Service, American Cancer Society and the Damon Runyon Memorial Fund for Cancer Research.

† The NDV was obtained through the courtesy of Dr. G. Sharpless of the Lederle laboratories, the PR8 strain from Dr. F. Horsfall of the Rockefeller Institute and the WS strain from Dr. H. Koprowski of Lederle laboratories.

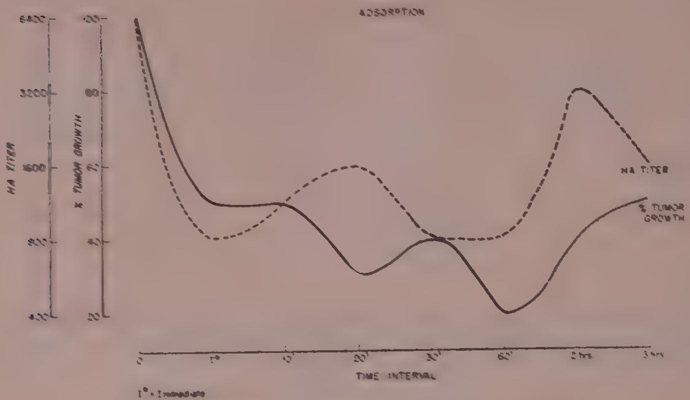

FIG. 1. Inhibition of sarcoma 180 by NDV at 4°.

centage of tumor growth regardless of the size of the tumor. In all experiments a comparable tumor suspension was mixed with normal allantoic fluid and the same procedures carried out. *Titrations.* All titrations for the hemagglutination (HA) of the virus were carried out by making two 1-10 dilutions in saline for the first two tubes and doubling dilutions thereafter. An equal volume (0.5 cc) 1/4% of washed adult chicken red blood cells in saline were added and the tests were read at the end of 1 hour at room temperature. Titrations for infectivity of the virus were made by inoculation of 0.2 cc of each dilution into the allantoic sac of three 10- or 11-day eggs. The dilution which killed ½ the embryos was taken as the end point and the result expressed logarithmically.

Experimental. The experiments will be presented under two headings, 1) adsorption of the virus as indicated by the fall in HA titer and as correlated with the failure of the tumor to grow and 2) elution of virus accompanied in most instances by an increase in HA and tumor growth. It is realized that absolute proof that the virus adheres to the tumor cells and results in their inability to grow is lacking and must await further work. For convenience of discussion, however, it is felt justifiable to carry over the terms used in discussing the hemagglutination of red cells by viruses.

Adsorption of NDV on sarcoma 180 tumor cell suspensions. 1) Effect of temperature. Experiments were carried out simultaneously at 4°C, room temperature which varied from 22-24°C, and 37°C. It was found that at 37° the control suspensions of tumor cells in normal allantoic fluid lost their viability so rapidly that no direct comparison could be made with the experiments done at the other temperatures. There was evidence, however, that the virus was adsorbed with inhibition of tumor growth. Undiluted NDV allantoic fluid was brought to the temperature employed in the experiment and then rapidly mixed with a 10% sarcoma 180 cell suspension (settled) at the same temperature. Directly thereafter

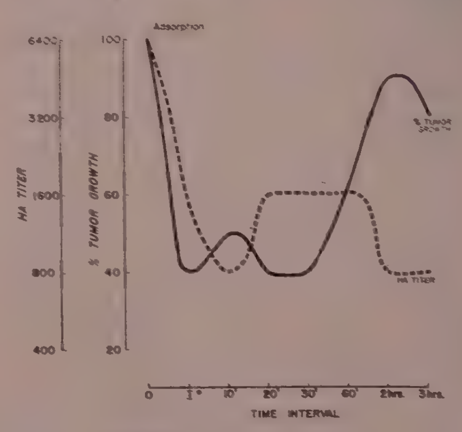

FIG. 2. Inhibition of Sarcoma 180 by NDV at room temperature.

6 cc was removed, 1 cc rapidly centrifuged to sediment the tumor cells and the remaining 5 cc was inoculated in 0.5 cc quantities into both flanks of 5 white mice. A hemagglutination titration was done on the centrifuged supernatant. This procedure was repeated at stated time intervals. As a control, another aliquot of tumor cell suspension was mixed with equal parts of normal allantoic fluid and mice were inoculated at the same time intervals. All of these tumors grew.

Fig. 1 and 2 show the result of a representative experiment. The tumor inhibitory effect was apparent immediately at both temperatures. It was maximum at 4°C in 60 minutes and at room temperature remained at its lowest level for 30 minutes, after which spontaneous elution, as measured by tumor growth, appeared to take place. The HA titer seldom coincided absolutely with the inhibition of tumor growth although the immediate fall in titer after mixing always occurred. No attempt was made to interpolate between the relatively large range of dilutions in the HA test. This might have resulted in a smoother curve. However, in experiments carried on for 8 to 12 hours it was found

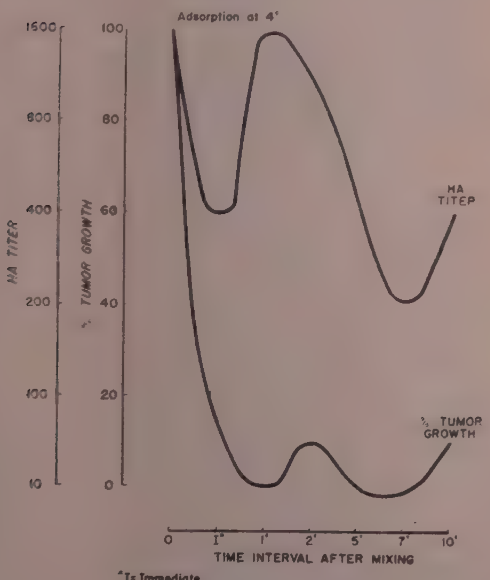

FIG. 3. Inhibition of growth of Sarcoma 180 suspensions by NVD.

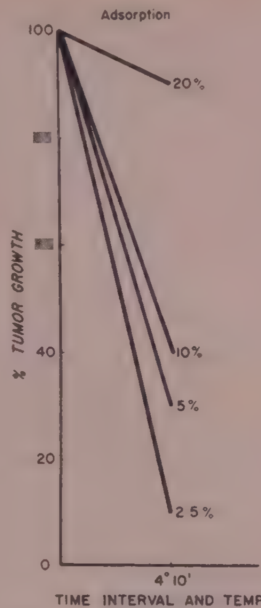

FIG. 4. Inhibition of Sarcoma 180 by NVD with different amounts of tumor suspension.

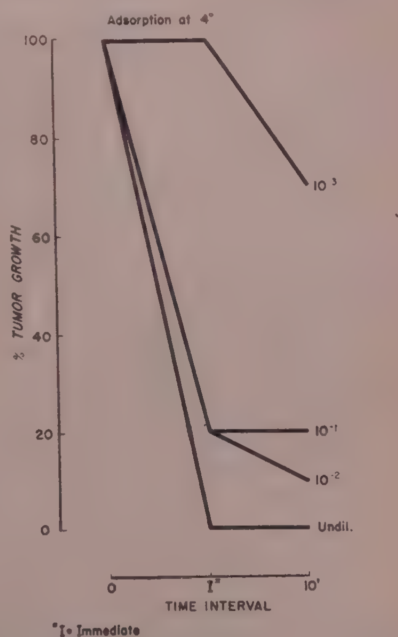

FIG. 5. Inhibition of sarcoma 180 by NDV with different dilutions of virus.

that the virus HA titer varied in an undulating fashion from high to low, whereas the inhibi-

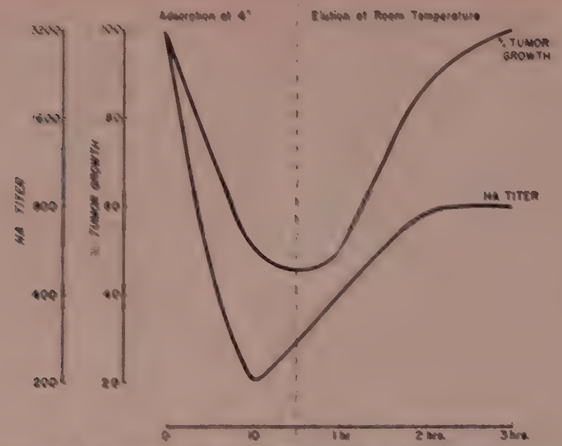

FIG. 6. Adsorption of Sarcoma 180 by NVD at 4° and elution at room temperature.

tion of tumor growth varied only slightly. In most experiments in which the effects of 4°C and of room temperature were compared the extent of tumor inhibition was approximately the same although some elution took place at room temperature.

2) Rapidity of the reaction. A sarcoma 180 suspension was settled for 10 minutes at 4°C to remove all the clumps. It was mixed in equal portions with NDV infected allantoic fluid and samples were removed at intervals of 1 minute for inoculation into mice and for determination of the HA titer. Fig. 3 shows the results of the experiment. It demonstrates that the inhibitory effect of the virus on the growth of the tumor cell takes place very rapidly.

3) Relation between the number of tumor cells and the inhibition of their growth. The sarcoma 180 suspension was made up in buffered Locke-Ringers to concentrations of 20%, 10%, 5% and 2.5%. Each concentration was mixed with an equal part of NDV infected allantoic fluid at 4°C. After 10 minutes mice were inoculated subcutaneously and a HA titer done on a sample of the supernatant. Fig. 4 shows that very little inhibition occurred with the 20% suspension but was demonstrable with all the others. The smaller the concentration of cells the more inhibition occurred. The control tumor suspensions grew in all instances. The HA titer of both the 20% suspension and 10% suspension fell from 1280 to 320, and for both the 5% and 2.5% suspension the fall was from 1280 to 160.

4) Influence of the amount of virus on tumor inhibition. NDV infected allantoic fluid was mixed with normal allantoic fluid to make dilutions of 10^{-1}, 10^{-2} and 10^{-3}. These, along with the undiluted fluid, were then mixed with an equal amount of settled sarcoma 180 suspension and adsorption carried out at 4°C. Mice were inoculated and HA titrations done immediately and after 10 minutes. The results given in Fig. 5 show that maximum tumor inhibition occurred with the undiluted NDV infected allantoic fluid

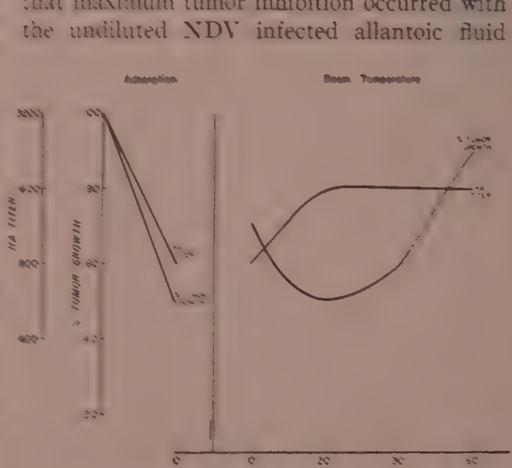

FIG. 7. Elution of NDV from tumor suspension at room temperature for a one hr period.

TABLE I. Persistence of NDV in Sarcoma 180 in the Mouse.

Time after inoc.	Titer
Immed.	7.5*
10 min	5
30 "	5
60 "	5
1 day	3
2 "	3
3 "	1
4 "	0

* Expressed as the reciprocal of the LD_{50}.

and was least with that diluted 1-1000. HA titers showed a drop concurrent with tumor inhibition except for the 10^{-3} dilution where no HA titer was demonstrable. Repeated experiments have given the same general result and show that there is a critical level beyond which the virus cannot be diluted to obtain the inhibitory effect.

Elution of NDV from sarcoma 180 suspension. After adsorption at 4°C for short periods as described above, it was found that placing the flask at room temperature for varying periods of time resulted in an increase in the hemagglutination titer and concurrent with this the tumor cells "regained" their ability to grow. Fig. 6 demonstrates the result of adsorption at 4°C for 10 minutes and subsequent elution at hourly intervals. In another experiment adsorption was carried out at 4°C for 10 minutes and then the flasks placed at room temperature for 1 hour. During this time aliquots were removed at regular intervals for inoculation of mice and for HA titers. The results are given in Fig. 7. It can be seen that elution does not take place until 1 hour and is not quite complete. Control tumor suspensions with normal allantoic fluid grew in all instances. The elution process is much slower than the adsorption and is not always demonstrable. The failure of elution is probably also related to the amount of virus and the number of cells. Further work is being done to elucidate this point.

Failure of the NDV to grow in the sarcoma 180. When NDV was inoculated in 0.1 cc quantities directly into the tumor of sarcoma 180 bearing animals it persisted but there was no evidence of multiplication, Table I. Animals were killed at regular intervals and the virus content of their tumors titered by egg inoculation. The experiment was carried out for 11 days and only traces of virus could be detected on the 8th and 9th days when 1 chick embryo out of 3 given a 10^{-1} dilution of tissue died and showed a positive HA. In addition, in tissue culture experiments in which virus was inoculated into minced sarcoma 180 in Tyrode's solution it was impossible to keep the virus living for more than 3 consecutive passages. Bioassays showed the tumor to be viable. Parallel cultures made in chick embryo showed that the virus multiplied readily in this medium. In summary, there is no evidence that the NDV multiplies in the sarcoma 180 whether in the intact animal or in tissue culture where the source of living cells is the sarcoma 180.

Effect of immune serum on the reaction. Immune serum was prepared by inoculation of rabbits with 10 cc of NDV infected allantoic fluid intravenously twice at weekly intervals. Normal allantoic fluid (NAF) was inoculated in the same way. Two weeks after the final inoculation the rabbits were bled and serum collected. Sarcoma 180 tumor cell suspensions were prepared in the usual manner and mixed with equal parts NDV infected allantoic fluid and placed at 4°C for 10 minutes for adsorption. HA titers and animal inoculations showed that adsorption had taken place, Table II. The cells were removed, centrifuged and washed in twice the volume of buffered Locke-Ringer solution. They were again sedimented and resuspended in fresh diluent to 5 cc. One cc of anti-NAF serum, buffer, pre-vaccination sera and sera from the vaccinated rabbit at different dilutions was added. The flasks were allowed to stand at room temperature for ½ hour after which mice were inoculated subcutaneously. It is apparent that the anti-NDV serum even when added in high dilution to the cell suspensions resulted in complete tumor growth in contrast to the cells to which the other sera and buffered Locke-Ringers had been added. Since the reaction can be inhibited by specific serum it indicates that the virus particle is responsible for the inhibition of tumor growth.

TABLE II. Effect of NDV Immune Serum on Inhibition of Tumor Growth by NDV.

	4° 10′		Resuspended S-180 at ½ hr RT°		
	HA	T.G., %*	Materials added to cells (1 cc)	HA	T.G., %*
Orig.	3200		Buffered Locke-Ringers	10	40
4° 10′	1600	60	Anti-NAF serum	0	50
			Pre-vaccination serum	0	60
			Anti-NDV serum—undil.	0	100
			" " " —10^{-1}	0	100
			" " " —10^{-2}	0	100

* T.G. = Tumor growth.

Experiments with other tumors and other viruses. Another tumor which lends itself to this type of experiment is the Ehrlich carcinoma which will cause subcutaneous tumors when inoculated subcutaneously or a fatal ascites when inoculated intraperitoneally. When a 10% suspension was made from a solid tumor and mixed with equal parts of NDV, tumor inhibition associated with a fall in hemagglutination titer occurred in the same manner as in the experiment with sarcoma 180 when these mixtures were inoculated either subcutaneously or intraperitoneally. Elution also took place in the same manner.

Other hemagglutinating viruses have been used with the sarcoma 180 suspensions. Preliminary experiments lead us to believe that when sufficient PR8 strain influenza virus is present, it has the same inhibitory effect in tumor growth as the NDV. WS strain of influenza virus appears also to produce a profound inhibitory effect on the sarcoma 180 cells suspensions. With this virus (WS) no elution has been noted under the same conditions in which it takes place with the NDV. All of the details of the PR8 and WS viruses have not been worked out as yet. They will be the subject of another communication.

Discussion. The results here presented seem to be quite analogous to the hemagglutination of red blood cells by the Influenza-NDV-Mumps groups of viruses. It has been impossible to demonstrate agglutination of the tumor cells themselves because of their tendency to agglutinate spontaneously. The differences so far appear to lie mainly in the fact that the cells involved are tumor cells which ordinarily have the power of reproducing themselves when implanted into the proper host. When brought in immediate contact with the virus they lose this ability but later on after a change in temperature from 4°C to room temperature they slowly "regain" their ability to grow. Since this is always associated with a fall in hemagglutination titer during the "adsorbing" phase, and usually followed by an increase in hemagglutination titer during the "elution" phase it is tempting to consider the two systems as due to the same process. Since we have not been able to demonstrate actual multiplication of the virus in the sarcoma 180 in the intact animal or in tissue culture it seems that the inhibition of tumor growth may be associated with the presence of virus on the tumor cell. This would be analogous to that seen with inactivated bacteriophage which also is capable of inhibiting bacterial growth during the "adsorption" stage(2). It is difficult to explain the ability of the tumor cells to grow again after elution if we postulate on actual entrance of the virus particle into the cell.

The points which are under investigation now and which it is hoped will elucidate the mechanism more fully are 1) investigation of the tumor cell itself following adsorption and elution, 2) the effects of virus which has undergone physical treatment such as ultraviolet and heat, 3) attempts at receptor removal by chemical or enzymatic means, 4) the role of non-specific inhibitors in this reaction.

Summary. 1. When mixtures of sarcoma 180 and active NDV allantoic fluid are mixed at 4°C and inoculated subcutaneously into mice an inhibition of tumor growth takes place. This is associated with a fall in hemagglutination titer. After the mixtures have been brought to room temperature for one to 3 hours subcutaneous inoculation re-

sults in tumor growth. This is usually associated with an increase in hemagglutination titer. 2. No evidence has been found for the multiplication of the virus in the sarcoma 180. The results are discussed as an analogy with the hemagglutination of red blood cells by the Influenza-Mumps-NDV group of viruses.

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Prompt Effect of Adrenal Stimulation on the Free Amino Acid Content of Rat Tissues. (19480)

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In studies involving the prolonged administration of the hormone preparation, it was found(1) that various endocrine factors influenced the free amino acid concentration of rat tissues. In connection with certain of our investigations it was important to know if these effects could be elicited upon short term treatment. We are reporting the effects within 2 hours of adrenocorticotropin and of various adrenal steroids on the free amino acid concentration of rat tissues.

Experimental. Male Sprague-Dawley rats, weighing approximately 160 g, were used in groups of 4 to 8 for each experiment. The hormone was administered subcutaneously after a preliminary fast of 16 hours and the animals were sacrificed 2 hours later. Picric acid filtrates of various tissues were immediately prepared and analyzed for amino acid content by the ninhydrin gasometric method (2). Analytical values are presented as mg of amino N/100 g of tissue, wet weight.

Results and discussion. The experimental results are presented in Table I. Deviations from normal are considered significant only if P <0.02(3) and, in the table, are in italics. It can be seen that, of the tissues studied, only liver, kidney, and adrenal showed a short term response to hormone administration. This is in contrast to the report of Friedberg and Greenberg(1) where it was shown that, following several days treatment, kidney showed no response and there was a sharp rise for plasma. The inertness of cortisone acetate probably might be explained on the basis of poor absorption from the site of injection. That the liver, kidney, and adrenal changes represent specific target organ responses is supported by the lack of significant alteration in plasma levels.

An unexpected finding was the marked change in the free amino acid content of the adrenal following adrenocorticotropin (P <0.001). In view of the ease and precision

TABLE I. Influence of Various Adrenal Hormones and of Adrenocorticotropin on Free Amino Acid of Tissue Concentration of Rat Tissues.

Treatment	Dose/ 100 g	Tissue Heart	Liver	Kidney	Spleen	Adrenal	Plasma
Control	—	24.1±1.56	22.7±1.03	26.8±.85	35 ± .59	43.3± .86	4.21±.24
Adrenocortical extract (Upjohn)	.2 ml	25 ± .74	*28.7*± .87	*33.3*±.58	37.4±1.34	44.8±1.73	5.40±.40
Desoxycorticosterone glucoside	.5 mg	24.7± .59	*27* ± .47	27.4±.91	34.5± .45	43.8±1.47	5.10±.48
Cortisone acetate	.5 "	27.5± .76	26.4±1.02	28.9±.37	34.6± .66	46.3±1.98	4.19±.31
Adrenocorticotropin	.01 "	27.7± .69	*28.8*±2.00	*30.2*±.46	36.5±1.10	*53.6*±1.38	4.91±.31

Values are presented as means ± stand. errors.

of these determinations it is conceivable that this approach might be used in devising an assay method for adrenocorticotropin.

Summary. The effect within 2 hours of adrenocorticotropin and adrenal hormone administration on the free amino acid content of rat tissues was determined. Liver and kidney showed a significant increase following administration of either hormone preparation and the adrenals showed an increase following adrenocorticotropin administration.

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Genetic Modification of Response to Spleen Shielding in Irradiated Mice.* (19481)

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Jacobson and his coworkers(1,2) have demonstrated that shielding of the exteriorized spleen will protect CF-1 mice against lethal doses of whole body x-radiation. Hematopoietic activity in the shielded spleen is greatly stimulated within the first 24 hours after irradiation and recovery of hematopoietic elements in the bone marrow occurs sooner than in sham-shielded irradiated controls. However, spleen shielding has reportedly been only partially protective in other species. Evidence is presented herein of a significant difference in response of two different inbred strains of mice, associated with apparent differences in histologic appearance of the shielded spleens after irradiation.

Procedures. Mice of strains A (Strong) and C57 black, of both sexes, were divided equally with respect to age (range 32-69 days) among 3 groups. One was kept intact and received a single whole-body dose of 550 r. Physical factors were 120 Kvp, 9 ma, 0.25 mm Cu + 1.0 mm Al added filter, 30 cm mouse-target distance, 32 r/min. The spleens of the other two groups were exteriorized under nembutal anesthesia and placed respectively in lead or paraffin shields during irradiation to the same dose, as described by Jacobson *et al.*(1). Some animals in these groups died during operation. Animals were maintained under identical laboratory conditions and had free access to Purina Laboratory Chow and water. One animal of each strain was sacrificed in each group at 3, 5, 7, 9, and 11 days after irradiation; complete autopsies were performed and the thymus, spleen, superficial lymph nodes, and liver were fixed in Bouin's fluid for histologic examination. In addition, sternal bone marrow smears were made and stained with Wright's and Giemsa's stains. The remaining animals were observed daily for deaths during the first 30 days after irradiation.

Results are summarized in Table I and Fig. 1. The earliest deaths among sham-shielded A mice occurred on the 4th day, whereas deaths among the intact irradiated controls were not observed until the 12th day. From this point on, however, cumulative curves of these 2 groups were parallel and final mortality was almost identical. A similar premature onset of death, without a greater final incidence, occurred in sham-shielded C57 black mice. Death started significantly earlier among all groups of this strain than among strain A animals. The 2 strains also differed in the greater effect of

* This investigation was supported by a sub-contract from the Naval Radiological Defense Laboratory, San Francisco Naval Shipyard.

TABLE I. Effect of Spleen-Shielding on Radiation Mortality in Two Strains of Mice.

Treatment group	Strain A 32-41* days	42-51	52-61	62-71	Total		Strain C57 Black 32-41 days	42-61	62-71	Total	
						%					%
Spleen shielded	0/7†	0/21	0/10	0/4	0/42	0	4/7	2/11	2/17	8/35	23
Sham "	6/7	18/22	5/10	3/4	32/43	74	6/6	11/16	5/18	22/41	54
Intact	6/8	15/19	7/11	3/7	34/45	76	8/8	14/19	3/16	26/44	59

* Age at time of exposure to single dose of 550 r.
† Expressed as: No. dying/No. irradiated.

age upon susceptibility among C57 blacks(3); mortality decreased only moderately with age among the albino mice.

Spleen shielding protected strain A mice completely against mortality after 550 r. In contrast, mortality in spleen-shielded C57 blacks attained 23%, despite the lower final death rate in controls of this strain.

Histologic examination revealed essentially the same degree of initial damage in the thymus glands, lymph nodes, and bone marrows in all groups of each strain. The shielded spleens of both strains showed distinct enlargement and increased cellularity within 3 days. Those of strain A seemed enlarged primarily by the proliferation of hematopoietic elements in the red pulp, with rather small Malpighian corpuscles, whereas the shielded spleens of the serially sacrificed C57 blacks showed approximately the same areas of enlarged white pulp and red pulp in cross-sections. The numbers of animals are too small, however, to convey more than a suggestive difference. Recovery of the lymph nodes and thymus glands was not apparently influenced by spleen-shielding under these conditions. Bone marrow preparations were not technically satisfactory for quantitative comparison of the 2 strains.

Summary. It appears that the protective effect of spleen-shielding in irradiated mice may be genetically conditioned, and related

EFFECT OF SPLEEN SHIELDING ON MORTALITY IN TWO STRAINS OF MICE

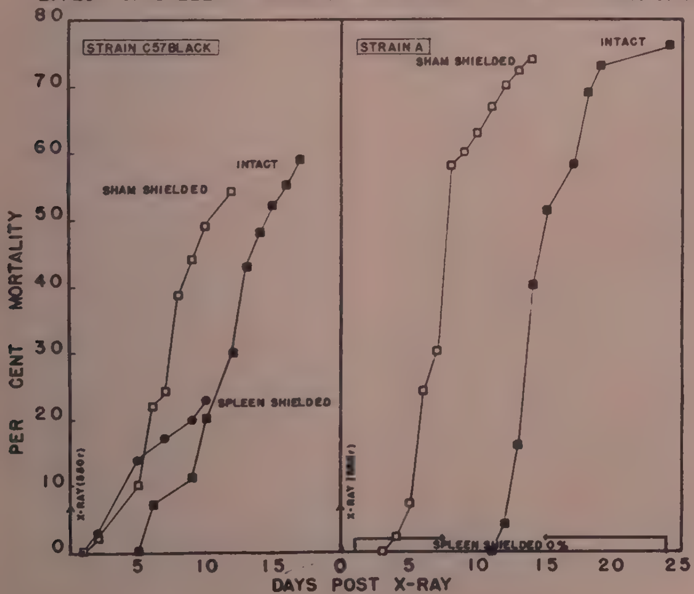

FIG. 1.

to the importance of the spleen in the hematopoietic system of each strain.

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Salicylates and Experimental Beryllium Poisoning. (19482)

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Acute beryllium poisoning in mice, readily produced by the intravenous injection of soluble beryllium salts, usually terminates fatally in 3 days(1,2). Death results from acute liver failure following hepatic focal and mid-zonal necrosis(1,3). Additional pathological changes in the kidney and spleen have also been described. Recently, aurintricarboxylic acid, a substance of relatively low toxicity, was found to be an effective antidote when it was administered to mice up to 8 hours after they had received LD_{95} amounts of $BeSO_4$ intravenously(2). This protection was attributed to its chelating and lake-forming properties. The present study was undertaken to ascertain if the salicylic acid portion of the aurintricarboxylic acid molecule would be as effective in reversing experimental beryllium intoxication.

Experimental. $BeSO_4$ was injected intravenously into young adult female CF#1 mice in an aqueous solution containing 0.07 mg Be per ml at a pH of 3.6-3.7. The animals were injected with 0.7 mg Be per kg, an amount which kills approximately 95% of the mice within 2 weeks. Compounds tested for antidotal properties were administered intravenously one hour later, except as otherwise noted. Control animals given only beryllium showed a 2.2% survival after 14 days with a 50% mortality time of 2.4 days.

Sodium salicylate was studied at various dose levels and the results, summarized in Table I, show this compound to be effective as an antidote when given in adequate doses either intravenously or intraperitoneally. Of the sodium salts of various analogues of salicylic acid tried, those without the ortho-hydroxy, carboxylic acid structure were uniformly ineffective. However, not all compounds with this grouping showed protective properties. Sodium *p*-aminosalicylate gave only equivocal results. Sodium gentisate, a product of salicylate metabolism(4), and its analogue, sodium 2,4-dihydroxybenzoate, were ineffective.

Disodium catechol disulfonate, which has been found to be effective in the treatment of experimental uranium poisoning(6), was partially successful.

Sodium salicylate afforded protection when treatment with 400 mg per kg was delayed 8 hours but not when treatment was postponed 24 hours. Pretreatment 8 hours before the beryllium was administered was ineffective, probably because of metabolism and excretion of salicylate during this period.

Discussion. The toxicity of beryllium to tissues has been ascribed by several investigators to its soluble, ionized form(1,5). Among the factors believed to be involved in effective antidotal action against beryllium ions is the chelating action of the ortho-hydroxy, carboxylic acid structure, which reduces the availability of the toxic ionized beryllium(7). Such chelation is presumed to occur with salicylate as well as with aurintricarboxylic acid, which in addition forms a stable lake. Undoubtedly, factors other than chelation are involved in successful protection against acute experimental beryllium poisoning. For example, on a molar basis about 35

TABLE I. Effect of Salicylate and Salicylate Analogs on Experimental Beryllium Poisoning in Mice.

Compound*	Dose level, mg/kg	No. mice	% survival at 14 days	Time to 50% death in days
Beryllium controls	—	90	2.2	2.4†
Sodium salicylate (Na o-hydroxybenzoate)	600	20	100‡	—
	400	39	79	—
	200	10	60	.
	40	10	0	2.5
Sodium salicylate (intraper.)	600	10	100	-
" p-aminosalicylate	660	10	20	2.9
	400	30	57	—
" m-hydroxybenzoate	600	10	40	2.4
" p-hydroxybenzoate	600	10	0	2.4
" o-chlorobenzoate	600	10	0	2.8
" gentisate (Na 2,5-dihydroxybenzoate)	660	10	0	2.4
	330	10	10	1.9
Sodium β-resorcylate (Na 2,4-dihydroxybenzoate)	660	10	0	1.4
Disodium catechol disulfonate (Na_2 1,2-dihydroxybenzene-3,5-disulfonic acid)	800	10	50	8.4
	400	10	50	13.4
	200	9	33	4.3
	80	10	20	2.4

* All compounds administered intrav. except as noted.
† Mean value of 9 experiments.
‡ No deaths after 60 days and 132 days, respectively, in 2 groups of 10 mice each.

times as much salicylic acid is required for antidotal action than aurintricarboxylic acid. Moreover, the amount of salicylic acid used (600 mg per kg) is roughly 75% of the median lethal dose while the comparable effective dose of aurintricarboxylic acid is about 11% of its LD_{50}. Whether suitable chelating compounds with or without lake-forming properties will be effective in the treatment of acute beryllium poisoning in man and further in the therapy of chronic beryllium granulomatosis needs to be established.

Although the ortho-hydroxy, carboxylic acid grouping is present in the dihydroxybenzoates, in the compounds tested the presence of the second hydroxyl group nullifies the antidotal action toward beryllium. To the extent that gentisic acid is a metabolite of salicylic acid (4 to 8% in humans(4)), the protective properties of the latter are diminished by salicylate degradation. The major metabolic products of salicylic acid are the conjugates with glycine and glucuronic acid, and the rates at which these are formed from beryllium-salicylate chelates are not known. Consequently, it cannot be predicted whether sodium salicylate would be therapeutically useful in human beryllium poisoning or whether it would be a hazard because of the translocation and possible release of toxic beryllium ions in more susceptible tissues.

Summary. Intravenously or intraperitoneally administered sodium salicylate in dose levels of 600 mg per kg is an effective antidote for acute beryllium poisoning in mice when given up to 8 hours after the administration of intravenous beryllium sulfate in LD_{95} amounts. Salicylate analogues without the ortho-hydroxy, carboxylic acid grouping were generally ineffective as were 2 dihydroxybenzoates.

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Electrophoretic Studies of the Mucin Fractions from the Human Gastric Juice.* (19483)

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Glass and Boyd have separated from the mucin fraction of human gastric juice two substances which they call mucoproteose and glandular mucoprotein. They have published evidence that mucoproteose derives from the surface epithelial cells, perhaps as a breakdown product of visible mucus, while mucoprotein appears to be a product of the neck glands of the fundus and corpus(1-4). Precipitated with acetone, mucoproteose forms brownish, slightly hydrated resin-like clumps which adhere to the walls of the test tube, while mucoprotein appears as a light flocculent opal colored, highly hydrated precipitate. After drying with acetone, mucoproteose forms a chalk-white amorphous substance, whereas mucoprotein forms greyish crystal-like particles. Mucoproteose remains soluble while mucoprotein is insoluble below pH 4.0 after previous precipitation with acetone. On alkaline hydrolysis mucoproteose yields only 3.9-4.2 mg % tyrosine, while mucoprotein yields 7.5 ± 0.65. The nitrogen content of the mucoproteose is much lower than that of mucoprotein (5.7-7.3% as compared to $12.6 \pm 0.44\%$ respectively). On the other hand, the content of reducing substances in mucoproteose fraction is much larger than that in glandular mucoprotein. Recently Dische and Osnos examined the polysaccharide composition of these two fractions(5). They found that mucoproteose is very rich in galactose and mannose and contains very little hexuronic acid, while mucoprotein contains hexosamine and hexuronic acid, but does not contain galactose or mannose.

The electrophoretic technic of Tiselius(6) offers further promise for the separation and identification of various mucin fractions. To date, electrophoretic studies of gastric secretory products have been reported only on pepsin of animal origin(7) and on proteins of canine gastric juice(8). The present study is concerned with the identification of the electrophoretic properties of the two mucous substances separated from the human gastric juice by the technic of Glass and Boyd(1).

Materials used for electrophoretic analysis. I. *Gastric mucoproteose from human gastric juice.* Samples of gastric juice were recovered directly from the stomach of Tom, a fistulous subject who has been described in detail elsewhere(9). Other samples were obtained from pooled gastric juices of normal individuals and patients with gastric hyperfunction and duodenal ulcer. After mucoprotein had been precipitated and removed by the technic of Glass and Boyd(1) mucoproteose was prepared from the supernatant liquid either by a) acetone precipitation, followed by washing several times with acetone-water and acetone, and drying in the fresh air at room temperature, or b) by lyophilization of the supernatant fluid, after it had been submitted to dialysis against cold running tap water for 24 hours, and distilled water for another 24 hours at 6°C. II. *Glandular mucoprotein from human gastric juice* was recovered from the pooled specimens because Tom's gastric juice was uniformly poor in mucoprotein.

The mucoprotein precipitate was either a) dried with acetone after washing several times with distilled water, or b) lyophilized by the dry ice technic after washing several times with dilute HCl and distilled water, or c) directly dissolved in the buffer for electrophoresis after being washed several times with distilled water. The gastric juices used for separation of mucoproteose and mucoprotein fractions were placed in the refrigerator immediately after collection, and processed as soon as possible afterwards. The lyophilized mucoprotein and mucoproteose were all fairly soluble in phosphate and veronal buffers.

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However, it was necessary to first dissolve the acetone-dried proteins in a small amount of dilute alkali before adding the buffer. III. *Commercial porcine mucin* from two sources (Winthrop-Stearns granular mucin and Wilson powdered mucin[†]) was also analyzed for purposes of comparison. These powdered preparations were ground in a mortar with ⅕ normal NaOH so that they could be brought into solution before adding the buffer. An attempt was made also to separate the "glandular mucoprotein" fraction from the commercial mucin by processing it by the technic mentioned above. IV. *Crystalline pepsin (Armour)*, prepared by the method of Northrop(10), lot 80802, was used for electrophoresis without further processing. It was fairly soluble in phosphate and acetate buffers. V. *Canine gastric juice* was collected after sham feeding in dogs with gastric fistulae[‡] and dialyzed for 48 hours against running tap and distilled water at 6°C, then lyophilized by the dry-ice technic. *Technic of electrophoresis.* The electrophoretic technic used was the moving boundary method(6), and the apparatus used for this study was a machine built to the specifications of L. G. Longsworth(11). The light source was the standard mercury lamp with a green filter, and photographic plates used were of the Panchromatic type. The light source was found to be unsatisfactory for use with solutions of high concentrations (above 2%) because of turbidity. It is hoped that this difficulty will be eliminated in future studies by the use of a source of infra-red emission and infrared sensitive photographic plates or film. The average variation in temperature during the course of any single experiment was 1°C, being less in most experiments (from 0.4 to 1.0°C) although in a few it varied up to 1.8°C. The current varied at the most 0.7 milliamperes. In all but three experiments (done on Armour pepsin) an ionic strength of 0.1 was used. In these three experiments

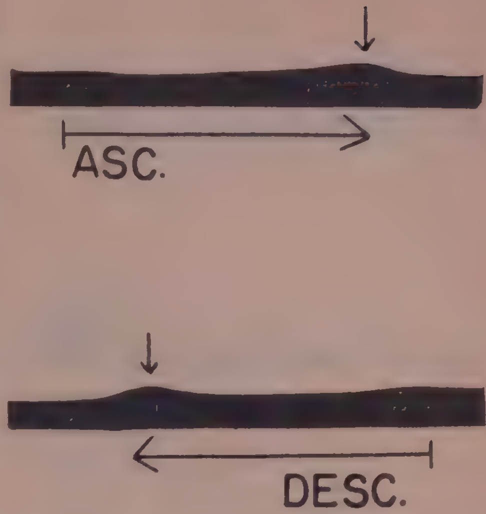

FIG. 1. Prepared mucoproteose from non-dialyzed human gastric juice run at pH 6.1. Note the broad ill-defined peak with a negative mobility of 5.51×10^{-5}, indicated by the vertical arrow. The center of the stationary boundary is indicated by the tail of the horizontal arrow in this and subsequent figures.

the ionic strength used was 0.05. Buffers used were acetate, phosphate and barbiturate (veronal), ranging from pH 3.7 to 8.5. The concentrations of pepsin used were from 0.6 to 1.0%, of glandular mucoprotein from 0.33 to 1.0% (usually 0.4-0.5), and for mucoproteose from 0.6 to 1.0%. The porcine mucins were run at the concentration of 1.0%, and the lyophilized canine gastric juice at the concentration from 1.0 to 1.5%. The potential gradient was from 4.37 to 8.57 volts/cm, and the time of run was in most instances from 2 to 5 hours with the exception of 5 shorter runs. The electrophoretic mobility of each component was calculated from its velocity, *i.e.*, the distance moved by the peak divided by the time, and the potential gradient, *i.e.*, the amperage divided by the average cross-sectional area of the cell and the electrical conductivity of the protein solution and buffer. In calculations of mobility in this study the distance moved by the peak (Δx) was measured from the descending limb pictures, with the exception of three runs in which it was necessary to make calculations from the ascending limb.

[†] Supplied kindly by Wilson Laboratories.

[‡] We gratefully acknowledge the assistance of Dr. W. L. Mersheimer, Department of Surgery, New York Medical College, who prepared the dogs with gastric fistulae.

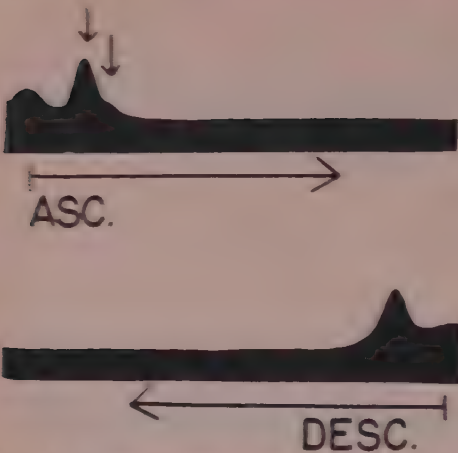

FIG. 2. Prepared mucoproteose, dialyzed and lyophilized, from human gastric juice run at pH 6.1. Note the fairly well defined peak of mobility −[illegible] × 10⁻⁵ (indicated by vertical arrow), and one or more poorly defined ones.

Results. Dissolved gastric mucoproteose from human gastric juice. Two preparations obtained from dried acetone precipitate and run at 0.6 and 1.0% concentration and pH 6.1 and 8.1 showed a very broad ill-defined peak (Fig. 1) with a negative mobility of 5.51 and 6.15 × 10^{-5} cm^2 $volt^{-1}$ sec^{-1}. Two other fractions of mucoproteose prepared by dialysis and lyophilization, and run at 0.6 to 1.0% concentration and at pH 6.1 and 8.5 showed one fairly well-defined, and one other poorly defined peak with negative mobility (Fig. 2). The well-defined peak of the dialyzed, lyophilized fraction at pH 6.1 was much slower (mobility −3.6 × 10^{-5}) than the peak seen at the same pH and concentration in the non-dialyzed acetone dried preparations. *Comment.* The marked difference in the electrophoretic pattern between the acetone dried preparation of mucoproteose and that prepared by dialysis and lyophilization may be attributable to denaturing of the material by the drying technic or to the loss of some of the components through dialysis.

Glandular mucoprotein from human gastric juice. Six samples from a single pool (A) were prepared by the acetone drying technic. The runs were done at a constant concentration of 0.4%, except in one case where a concentration of 0.33% had to be used, because of lack of material. The pH range varied in separate determinations from 4.6 to 8.6 in acetate, phosphate and veronal buffers. Three other mucoprotein samples prepared by the same technic (Pools B, C, and D) were run at a concentration 0.5-1.0% and at pH from 5.0 to 6.4. Another one (No. 2) was prepared by lyophilization at a concentration of 0.5% and pH 5.0, and 2 other samples (from Pools E and F) were prepared by technic (c) and run at pH 6.0 and 8.5 at concentration 0.5 and 0.4% respectively. In 11 out of the 12 samples of glandular mucoprotein tested, obtained from various pools and run over a pH range from 4.6 to 8.6, a single peak was obtained. This appeared fairly symmetrical and negatively charged throughout the range of pH tested. In one of the preparations (No. 2) which was run at pH 5.0 a small additional peak with a negative mobility was observed. This was much slower moving than the larger peak, and may have been due to contamination of the preparation by other protein from gastric juice (mucoproteose?). One of the typical electrophoretic patterns of glandular mucoprotein obtained is shown in Fig. 3. The mobility of glandular mucoprotein was found

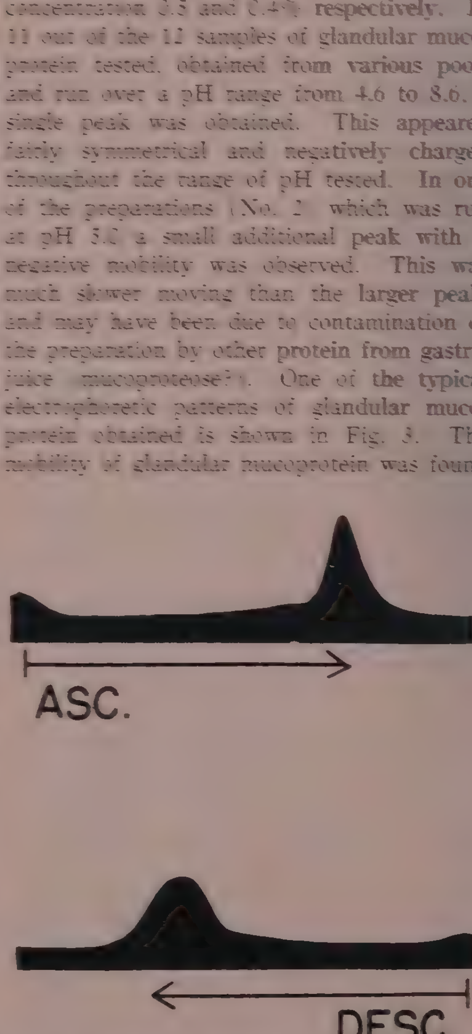

FIG. 3. Prepared glandular mucoprotein from human gastric juice run at pH 4.4. A single peak appeared with mobility of −7.2 × 10^{-5}.

TABLE I.

No.	Source	Preparation	Concentration, %	pH	Buffer	Elect. field strength	Time of run, sec	Electrophoretic mobility × 10^{-5}			
					Human mucoproteose						
1.	Pool A	Method a	.6	6	Phosphate	5.51	17100	−5.1			
2.	P. A. pat.	a	1	6.1		6.13	11940	−6			
3.	Pool B	b	.6	6.1		6.52	13020	− .6			
4.	B	b	1	8.5	Veronal	[illegible]	[illegible]	[illegible]			
					Human mucoprotein						
1.	Pool A	a	.4	4.6	Acetate	5.29	10500	−4.1			
2.	Ulcer pat.	b	.6	5		5.26	9420	−7.2			
3.	Pool B	a	.5	5		5.21	11970	−6.7			
4.	C	a	1	6		6.67	4590	−8.5			
5.	A	a	.4	6		[illegible]	[illegible]	[illegible]			
6.	F	c	.55	6	Phosphate	[illegible]	[illegible]	[illegible]			
7.	D	a	1	6.4		6.74	9480	−7.2			
8.	A	a	.4	6.4		6.88	10260	−8.4			
9.	A	a	.4	7.3		7.29	9330	−8.7			
10.	E	c	.4	8.5	Veronal	6.15	12360	−7			
11.	A	a	.33	8.5		4.37	10590	−8.4			
12.	A	a	.4	8.6		5.78	11250	−7.3			
13.	Mucoprotein added to Tom's gastric juice (total conc. 1.33%)		.33	6	Phosphate	5.44	9345	−9.2			
					Porcine mucin			Peak I	II	III	IV
1.	Gran.		1	7.9	Phosphate	7.35	8040	−1.4			
2.	Wilson		1	6		6.40	2910	−13.5	−7.3	−3	−.9
3.	Gran. mucoprotein		1	8		5.05	5490	−18 ‡			
					Whole canine gastric juice			Peak I	II	III	IV
1.	Pool A		1.5	6	Phosphate	6.58	8220	−10	−7.6	−5.3	−.5
2.	B		1	8.5	Veronal	6.04	3600	†	−8.3	−4.6	−.2
					Pepsin						
1.	Armour's com'l lot 80802		.6	3.7	Acetate	8.24	17580	−3.3*			
2.			.6	4.1		7.45	12960	−6.1*‡			
3.			.6	4.1		8.32	12180	−5.5*			
4.			1	5.5	Phosphate	6.12	10500	−8.9			
5.			1	6.1		6.96	6780	−10.6			
6.			1	6.1		5.73	8430	−11.6			
7.			1	6.4		6.92	7200	−10.9			
8.			.6	6.7		8.57	4380	−8			
9.			1	6.8		8.54	2460	−7.9			

* Ionic strength = .05. † [illegible] ‡ [illegible]

to be very fast, so that in phosphate buffers at pH 6.0 and concentration 0.4-1.0% it was about -8.5×10^{-5} cm^2 $volt^{-1}$ sec^{-1}. The range of mobility of mucoprotein in various samples studied and run at a concentration from 0.4 to 1.0% was from −7.2 to -8.6×10^{-5} cm^2 $volt^{-1}$ sec^{-1} over the range of pH from 6.0 to 7.3. The mobility of mucoprotein in veronal buffer at pH 8.5-8.6 was surprisingly similar to that observed in phosphate buffers over the range of pH 6.0 to 7.3. To ascertain whether the peak of glandular mucoprotein could be recognized on electrophoresis of a sample of whole gastric juice, the following experiment was done. Tom's whole gastric juice which did not contain any mucoprotein was lyophilized after dialysis, and submitted to electrophoresis at 1.3% concentration in phosphate buffer of pH 6.0. Several small peaks with negative mobilities were evident, but all of them were much slower than those of mucoprotein. Thereafter, glandular muco-

protein from one of the pools was added at a concentration of 0.33% to the same (Tom's) gastric juice preparation and the run was repeated at the same pH. A new peak appeared, the mobility of which was 9.2 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}, and which therefore fell in the range observed in mucoprotein preparations. Detailed data are listed in Table I.

Attempted identification of pepsin peak. Since the technic used in separating mucoprotein from gastric juice by precipitation at a pH of 2.0 leaves pepsin in the supernatant fluid an attempt was made to prepare the mucoprotein fraction in another way so that it could contain pepsin as a contaminant. This was done on two samples of gastric juice rich in pepsin, by precipitating mucoprotein at a pH of 3.5 (at which pepsin appears in the protein precipitate). Electrophoretic study of these mucoprotein fractions, however, showed again only one single peak whose mobility at pH 8.5 in veronal buffer was similar to that of other mucoprotein samples, but which in phosphate buffer at pH 6.0 (calculated from the ascending limb) seemed to be higher than that of remaining mucoprotein samples.

Porcine mucin. The electrophoretic pattern of Wilson powdered commercial hog mucin was determined at pH 6.0 and concentration 1%. One very fast moving peak of a mobility –13.5 x 10^{-5} and 3 slower moving peaks (–7.3, –3.0 and –0.9 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}) were found. The slowest peak was the largest one. The electrophoretic pattern of the Winthrop-Stearns granular hog mucin was studied at pH 7.9 and in 1.0% concentration. One slow moving peak with a negative mobility of –1.2 x 10^{-5} was found, but it was strongly suspected that another fast moving peak was also present, but was "lost" when the experiment was left unobserved for two hours. From the same mucin a "glandular mucoprotein fraction" was prepared by the above-mentioned technic(1). The electrophoresis was done at pH 8.0 and in concentration of 1.0%. A single very fast moving peak was found with a very high negative mobility of –18.0 x 10^{-5} (if calculated from the ascending limb).

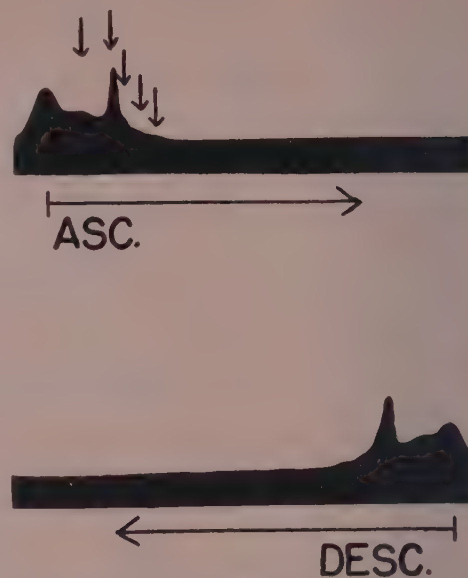

FIG. 4. Whole canine gastric juice, dialyzed and lyophilized, at pH 8.5. There are approximately 5 peaks of mobility ranging from –8.3 to –.2 × 10^{-5}.

Canine gastric juice. Four or more peaks were observed in the electrophoretic pattern of the canine gastric juice which was dialyzed and lyophilized and run at a concentration of 1.0-1.5% and a pH 6.0 and 8.5. In Fig. 4 one of these electrophoretic patterns is shown. The small first and second peaks are somewhat faster than peaks observed by Grossberg *et al.*(8), the third and the fourth (largest) peaks exhibit mobility corresponding exactly to F-1 and F-2 fractions as described by these authors. However, the fifth peak is slower than the slowest peak observed by these authors.

Crystalline Northrop pepsin from porcine origin (Armour). Nine electrophoretic runs were done on this material at a concentration from 0.6 to 1.0% and over the range of pH from 3.7 to 6.8 in acetate and phosphate buffers. All electrophoretic patterns showed one single large peak with a negative mobility, which being calculated at pH 5.5 was –8.9 x 10^{-5}, and at pH 6.1 was from 10.6 to 11.6 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}. This wide variation in speed is unexplained but may be the result of using the crude preparation without further

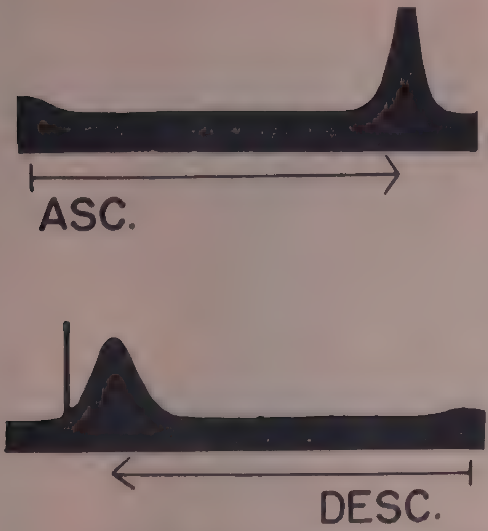

FIG. 5. Crystalline pepsin (Northrop) of porcine origin run at pH 5.5. One single large peak is seen with mobility of -8.9×10^{-5}.

purification. These mobilities are only slightly higher than those observed in similar preparations by Grossberg *et al.*(8). They are, however, definitely higher than the mobility of the glandular mucoprotein from human sources. The electrophoretic pattern of porcine pepsin is shown in Fig. 5.

Discussion. These electrophoretic data appear to confirm the earlier inferences of Glass and Boyd that mucoproteose and glandular mucoprotein are two quite separate components of the gastric juice and that mucoprotein is a relatively homogeneous substance. The various peaks of the mucoproteose fraction move more slowly than that of mucoprotein. The very fast moving peak observed in the specimens of Wilson porcine mucin may correspond to human glandular mucoprotein while the granular hog mucin preparation which is mainly surface mucus was characterized by a much slower peak which may correspond to human mucoproteose.

The electrophoretic method cannot settle definitely the question as to whether or not glandular mucoprotein is a single chemical substance. Although the appearance of the peak on electrophoresis is fairly compatible with homogeneity, it is not possible to exclude contamination of this component with other substances present in small concentrations. This is an important consideration since recent evidence indicates that glandular mucoprotein is the main carrier of Castle's intrinsic factor or even may be identical with the latter(12).

The failure to identify a peak of pepsin in human gastric juice is in keeping with the findings of Grossberg *et al.*(8) who also were unable to obtain a separate pepsin peak in their samples of canine gastric juice. Whether pepsin is adsorbed in one or more of the mucin fractions, or whether it is bound mainly to glandular mucoprotein, or has as yet an unidentified peak of its own is currently being studied, as are the electrophoretic patterns of the whole human gastric juice(12).

Summary. 1. Electrophoretic analysis was performed on 2 prepared constituents of dissolved mucin from human gastric juice, *i.e.*, glandular mucoprotein from gastric glands and dissolved mucoproteose from surface epithelium as separated by the technic of Glass and Boyd. The patterns obtained from human sources were compared with those obtained on electrophoresis of crystalline porcine pepsin, commercially available hog mucin, and lyophilized and dialyzed canine gastric juice. The runs were done over a wide range of pH and various preparations of these materials were studied with the moving boundary technic. 2. It was found that the electrophoretic pattern of mucoproteose with peaks of slow negative mobility was distinct from that of glandular mucoprotein which displayed a slightly asymmetrical single peak of fast negative mobility. It seems reasonable to conclude that these two substances represent separate constituents of the gastric mucus. Their possible functions will be the subject of later study.

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Histochemical Demonstration of Esterase Activity in the Normal Human Kidney and in Renal Carcinoma.* (19484)

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With the method of Gomori(1) esterase activity could be demonstrated in paraffin sections of various animals(2,3) but not in the human kidney(2,4,5). Some irregular staining is occasionally seen, however, in paraffin sections in which esterase activity is demonstrated by the technic of Nachlas and Seligman(6) using beta naphtyl acetate as substrate(7). It is the purpose of this communication to point out the fact that with both methods esterase activity can be regularly visualized in the normal human kidney as well as in some renal carcinomas if one works with frozen sections instead of paraffin sections.

Method. The material used consisted of 20 kidneys obtained at autopsy and 10 surgically removed specimens. The age of the subjects from which the kidneys had been obtained varied from a few hours to 70 years. Seven renal carcinomas were also studied. Frozen sections of unfixed tissues were cut at 10 to 15 μ. Frozen sections may also be prepared from tissues that have been fixed in acetone at 4°C for 24 hours or in 10% formalin for 3 hours at room temperature. Esterase activity in these sections can then be demonstrated with either the technic of Gomori or the dye method of Nachlas and Seligman. According to Gomori's method sections were incubated for 18 to 20 hours with Tween 40 as substrate. After developing the sites of enzymatic activity by treatment with diluted hydrogen sulphide, the sections were kept for several hours in 10% formalin and mounted in glycerogel. These preparations could be preserved for several months. Sections stained with the dye method had to be studied rapidly since the color fades within a few minutes. All attempted modifications did not alter this disadvantageous condition.

Results. The localization of esterase activity in frozen sections of human kidney not involved by disease with both methods was similar, although the dye method gave more uniform results. Within the cortex esterase activity was present in the cytoplasm of both the proximal and distal convoluted tubules (Fig. 1). Slight granular deposits were also noted in glomeruli but their significance appears doubtful. Some tubules in the medulla also showed esterase activity. From their location and caliber they are presumed to be straight portions of the proximal convoluted tubules and ascending portions of Henle's loop. The renal tumors studied were carcinomas of the clear cell and papillary types. No esterase activity was demonstrable in paraffin sections. In frozen sections, however, neoplastic cells showed a varying degree of

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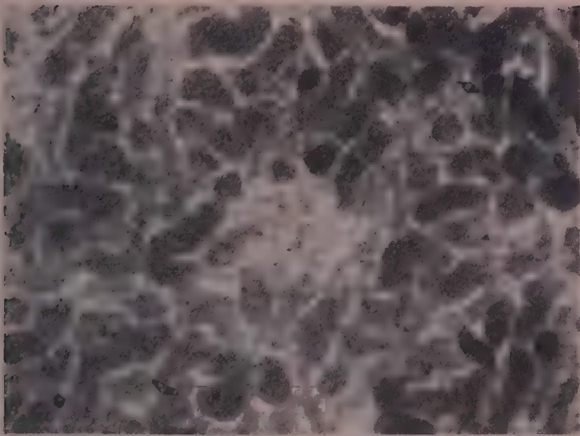

FIG. 1. Kidney cortex of human. Fresh frozen section. Gomori's method for esterase activity. Marked esterase activity is seen in the proximal and distal convoluted tubules. A glomerulus in the center is almost devoid of the lead sulphide deposits which indicate enzymatic activity. × 100.

FIG. 2. Renal carcinoma of papillary type. Fresh frozen section. Gomori's method for esterase. Considerable esterase activity is seen in the cells composing the tumor. × 100.

cytoplasmic activity (Fig. 2) in 5 of the 7 carcinomas. Esterase activity was seen in paraffin sections of only a few of many examined carcinomas by Gomori(2) and in several bronchus carcinomas by Menk and Hyer(8).

Comment. The recorded observations make it quite obvious that the process of dehydrating and embedding destroys most of the histochemically demonstrable esterase activity in the human kidney. The latter on chemical examination contains a lower average amount of esterase than the kidney of mouse, rat, guinea pig, rabbit, and dog(9). Since in paraffin sections, however, remnants of esterase activity can be demonstrated with beta naphtyl acetate as substrate, the azo dye method appears somewhat more sensitive than Gomori's technic. It is thus apparent that an important source of error in the histochemical esterase technic is caused by the loss of enzyme during the process of histological preparation. Some of the previously reported negative findings and inconsistencies(5) using paraffin embedded material must be re-evaluated with frozen sections. Formalin fixation, especially for longer periods of time(10) should probably be avoided especially if only small amounts of esterase are present since it

also leads to considerable inactivation of the enzymes(11). Barnett and Seligman(12) have recently introduced 2 more substrates, Indoxyl acetate and Indoxyl butyrate, for the histochemical demonstration of tissue esterase which do not diffuse into lipoids as the azo dye will do in fresh frozen sections. These authors stressed the usefulness of the Coons modification(13) of the Linderstrøm-Lang and Mogensen technic(14) for the preparation of thin frozen sections of various organs well suited for histochemical enzyme studies.

Summary. Esterase activity in the human kidney is not visualized in paraffin sections of acetone fixed tissues with Gomori's technic and only very irregularly with the azo dye method. It can be regularly demonstrated with both methods in fresh frozen sections. In 5 of 7 renal carcinomas esterase was found in the cytoplasm of tumor cells if fresh frozen sections were used but not in paraffin fixed material.

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Calcification VIII. Glycolytic Enzymes and Phosphorylated Intermediates In Preosseous Cartilage.* (19485)

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At present there is direct evidence for the existence of 4 components of the glycolytic cycle in preosseous cartilage, namely, glycogen (1,2), phosphorylase(3), lactic acid(4), and adenosine triphosphate(5). Additional evidence of the glycolytic system in preosseous cartilage comes from the use of glycolytic inhibitors(6). These agents inhibit *in vitro* calcification(6,7), which is restored by phosphorylated intermediates beyond the locus of action of the enzymes inhibited. The suggestion was made from these studies(3,7) that a glycolytic system is present in preosseous cartilage. However, while the use of inhibitors is suggestive, it is not conclusive evidence, since the degree of specificity of the inhibitors is not well established(8).

The purpose of this investigation, therefore, was to seek more direct evidence for the existence of other components of the glycolytic system in preosseous cartilage.

Procedure. Most of the enzymes to be described in cartilage have been demonstrated using the procedures of Racker(9). These depend upon the enzymatic oxidation of reduced diphosphopyridine (DPN) in the spectrophotometer. For this purpose, aqueous extracts of rachitic bone cartilage were employed. 250 to 400 mg of cartilage obtained from the metaphyses of the tibiae of rachitic

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Wistar rats† was crushed and extracted three times with 3 ml of cold distilled water. The extracts were combined and made up to 10 ml. Phosphorylated intermediates in the glycolytic cycle were detected in the following way: rats were injected intraperitoneally with approximately 15 million counts of radioactive phosphorus as inorganic phosphate. They were sacrificed after approximately 24 hours. The tibiae were removed as before and extracted, 3 times with cold 10% trichloracetic acid (TCA). These were spotted on Whatman No. 1 filter paper and chromatographed by downward migration in an ethanol-acetic acid developer (80 ml ethanol, 0.8 ml acetic acid; mixture then brought to pH 3.5 with hydrochloric acid, after which distilled water is added to give a final volume of 100 ml) using a Chromatocab.§ At the end of a 15-hour interval, the chromatograms were removed, dried, and placed in contact with x-ray film for 48-72 hours. The position of the individual compounds which had become radioactive was indicated by film exposure. This procedure is essentially that used by Benson *et al.*, in studies on photosynthesis (10), and by Cohen *et al.*(11). The spots were identified by chromatographing pure compounds under the same conditions and detecting these by chemical means.‡

Results and discussion. The first enzyme investigated was *aldolase*. Goldenberg and Sobel(12) obtained evidence for this enzyme by the colorimetric estimation of the 2,4-dinitrophenylhydrazine derivatives of the carbonyl groups liberated by the enzymatic degradation of hexosediphosphate (HDP)

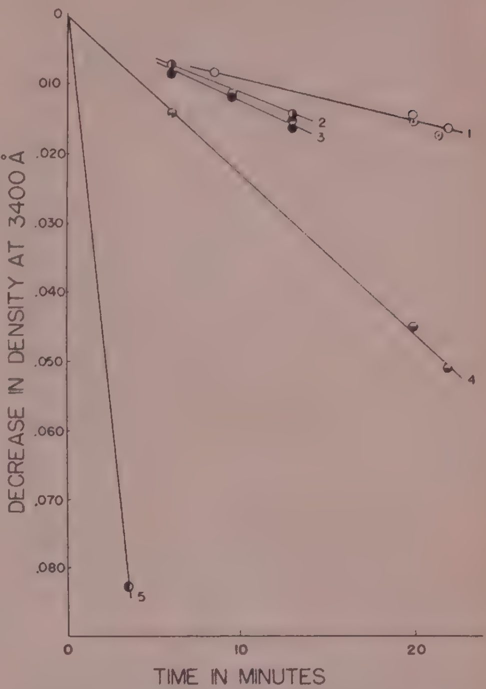

FIG. 1. Oxidation of reduced DPN by extracts of rat cartilage in presence of different substrates. Each cuvette contained .5 ml rat extract, .2 ml .1 M ammonium phosphate buffer pH 7.6, .1 ml .002 M reduced DPN, substrate, and water to 3 ml.

Curve 1: No substrate ○; no substrate, .2 ml 7×10^{-4} M ATP (dot in circle). Curve 2: .1 ml .25 M glucose, .2 ml 7×10^{-4} M ATP (solid right side circle). Curve 3: .1 ml .0058 M glucose-6-phosphate, .2 ml 7×10^{-4} M ATP (solid top half circle); .1 ml .0058 M fructose-6-phosphate, .2 ml 7×10^{-4} M ATP ●. Curve 4: .2 ml 1.6×10^{-4} M HDP (solid bottom half circle). Curve 5: .2 ml .035 M pyruvate (solid left side circle).

(13). Evidence for aldolase and α-glycerophosphate dehydrogenase was obtained by incubating the aqueous extract with HDP, reduced DPN, and ammonium phosphate buffer, pH 7.6, in the quartz cells placed in the spectrophotometer set at 3400 Å. If HDP is split to dihydroxyacetone phosphate and 3-phosphoglyceraldehyde, oxidation of reduced DPN can only occur via the conversion of dihydroxyacetone phosphate to α-glycerophosphate. The results of a typical experiment are shown in Fig. 1 (curves 1 and 4). There is a small decrease in density in the control cuvette (curve 1), which contains everything but HDP, and a

† The authors are indebted to Miss Mona Oser and to Dr. B. Oser of Food Research Laboratories, Long Island City, for generously providing many of the rachitic rats used in these studies. We wish to thank Dr. Harry Goldenberg of the Department of Biochemistry, Jewish Hospital of Brooklyn for many helpful suggestions.

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‡ Pure phosphorylated compounds for use in enzyme experiments as well as chromatographic runs were obtained from the Schwarz Laboratories, New York City, and the Ernst Bischoff Co., Ivoryton, Conn. Phosphopyruvate, as the silver salt was a gift from Dr. Gerhardt Schmidt.

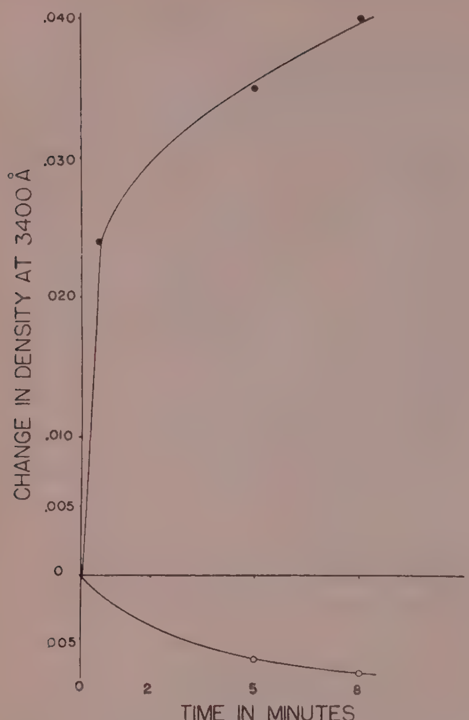

FIG. 2. Reduction of oxidized DPN by extracts of rat cartilage in presence and absence of α-glycerophosphate. Each cuvette contained .5 ml rat extract; .1 ml 3×10^{-4} M oxidized DPN; .2 ml .1 M pyrophosphate buffer pH 8.8; .3 ml 30% α-glycerophosphate and water to 3 ml. Lower curve, no substrate; upper curve with substrate.

rather rapid change in the presence of the substrate. These results point to the presence of aldolase and α-glycerophosphate dehydrogenase. Direct evidence for the presence of α-glycerophosphate dehydrogenase was obtained by starting with α-glycerophosphate and measuring the reduction of oxidized DPN in the spectrophotometer. The results of such an experiment are shown in Fig. 2.

It is not possible to demonstrate triosephosphate dehydrogenase in the presence of α-glycerophosphate dehydrogenase spectrophotometrically. If cartilage contains triosephosphate dehydrogenase, its presence can be detected in the following way: Using HDP as a source of 3-phosphoglyceraldehyde and oxidized DPN, the activity of the enzyme would lead to the formation of phosphoglyceric acid and reduced DPN. By adding an excess of pyruvate and lactic dehydrogenase, lactic acid would accumulate and this could be measured directly. The results of such an experiment are shown in Table I. The formation of lactic acid does not take place to any great extent in the absence of substrate, not in the presence of 0.0033 M iodoacetate. This first group of experiments has proven that aldolase, α-glycerophosphate dehydrogenase, and triosephosphate dehydrogenase are present in extracts of cartilage.

The presence of enolase in these extracts can be checked by using 2-phosphoglyceric acid as a substrate and reduced DPN. Results of such an experiment are shown in Fig. 3 along with the same system in the presence of fluoride. Phosphoglyceric acid in this case would be converted to phosphoenolpyruvic acid and then to pyruvic acid. Pyruvate in turn would be reduced to lactate, and reduced DPN oxidized, thus accounting for the change in density at 3400 Å. More direct evidence of the presence of enolase may be obtained by measuring the formation of phosphoenolpyruvate from 2-phosphopyruvate directly. This can be measured since the appearance of phosphoenolpyruvic acid is accompanied by an increase in density at 2400 Å (Fig. 4)(14). In the initial formulation of the enolase experiment described above, the assumption was made that a lactic dehydrogenase is present. This can also be proven directly by using

TABLE I. Triosephosphate Dehydrogenase Activity of Rat Cartilage.

Test system contains:	ml
$KHCO_3$ (.4 M)	.2
HDP (.15 M)	.1
Na arsenate (.15 M)	.1
Na pyruvate (.54 M)	.1
Lactic dehydrogenase (1 mg 50-70 fraction from rabbit muscle)	.1
Na F (1 M)	.1
Cysteine (.09 M)	.1
Cartilage extract	.5
Water	to 1.7

At end of 1 hr, add 1.5 ml 10% TCA; centrifuge; 1 ml used for lactic acid determination.

μg of lactic acid formed:	
(1) Complete system	55.6
(2) Complete system in presence of .0033 M iodoacetate	27.9
(3) Complete system without HDP	24

pyruvate as a sole substrate in the presence of reduced DPN. That this enzyme is present is shown by the data in Fig. 1 (curve 5).

We have thus far demonstrated several of the enzymes in glycolysis: aldolase, α-glycerophosphate dehydrogenase, triose phosphate dehydrogenase, enolase, and lactic dehydrogenase. The question then arose as to the presence of enzymes which convert glucose to hexose diphosphate. Fructose-6-phosphate and ATP, when added to tissue extracts, lead to the oxidation of reduced DPN. This means that hexosediphosphate is formed and that a phosphohexokinase is present (Fig. 1). Phosphohexoisomerase activity was demonstrated by the fact that glucose-6-phosphate in the presence of ATP leads to the oxidation of reduced DPN (Fig. 1). Finally, hexokinase activity is indicated by the addition of glucose and ATP as sole substrates and the subsequent oxidation of reduced DPN (Fig. 1, curve 2).

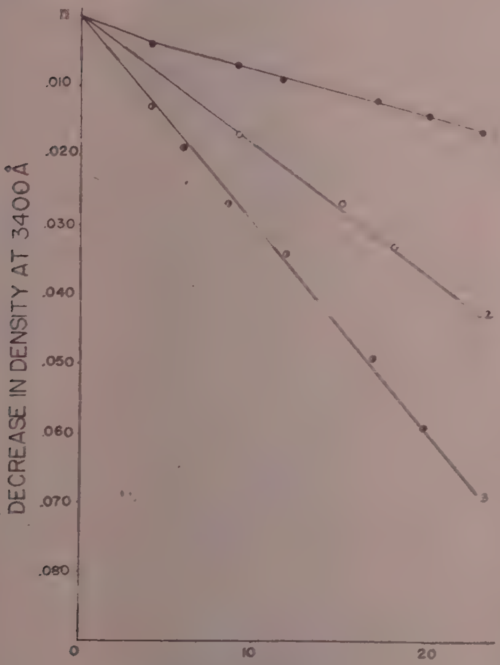

FIG. 3. Oxidation of reduced DPN by extracts of rat cartilage in presence of phosphoglyceric acid and fluoride. Each cuvette contained, except where otherwise indicated. .5 ml rat extract; .2 ml 8.5 × 10^{-4} M ADP; .2 ml .016 M phosphoglyceric acid: .1 ml .002 M reduced DPN; .2 ml .1 M ammonium phosphate buffer pH 7.6: .3 ml .1 M sodium fluoride; water to 3 ml. Curve 1: No fluoride, no substrate ●. Curve 2: With fluoride and substrate ○. Curve 3: No fluoride, with substrate (solid right side circle).

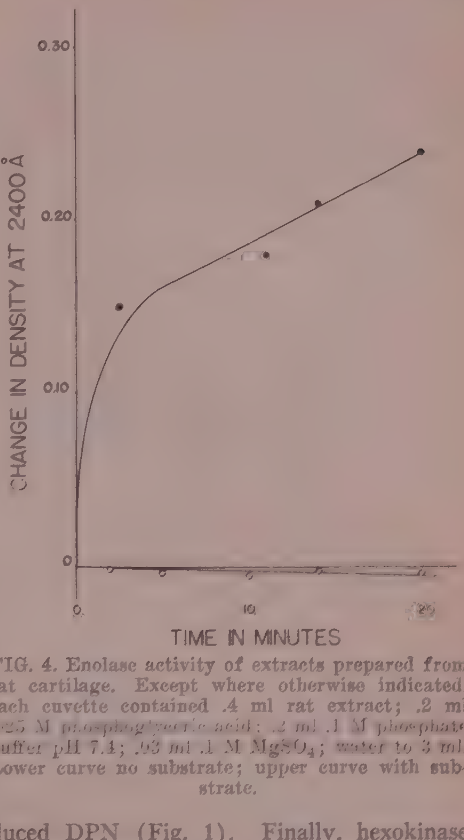

FIG. 4. Enolase activity of extracts prepared from rat cartilage. Except where otherwise indicated, each cuvette contained .4 ml rat extract; .2 ml .025 M phosphoglyceric acid; .2 ml .1 M phosphate buffer pH 7.4; .03 ml .1 M $MgSO_4$; water to 3 ml. Lower curve no substrate; upper curve with substrate.

If cartilage possesses a glycolytic system, one should also expect to find those of the phosphorylated intermediates which accumulate to some extent. We have been able to demonstrate some of the intermediates, as

TABLE II. Rf Values for Pure Compounds and Compounds Present in Trichloracetic Acid Filtrates of Epiphyseal Cartilage.

Compound	Rf for pure compound	Rf for tissue extract
Hexose diphosphate	.17	.163 (5)
Phosphoglyceric acid	.21	.224 (5)
Glucose-1-phosphate	.30	.299 (9)
Fructose-6-phosphate	.46	.465 (5)
Inorganic orthophosphate	.44	.439 (9)
Phosphopyruvic acid	.62	.614 (11)

Figures in parentheses indicate number of determinations used to obtain average value for Rf.

shown in Table II, after injecting radioactive phosphorus and preparing TCA filtrates.

Summary. 1. Most of the enzymes of glycolysis present in muscle and yeast appear to be present in epiphyseal cartilage. 2. Several of the phosphorylated intermediates of the cycle have been identified using chromatographic procedure.

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Further Studies on Relationship Between Human Serum Cholinesterase and Serum Albumin.* (19486)

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Of the two types of cholinesterase which have been described for the human, one, the specific or "true" cholinesterase, has received most of the emphasis in research because of its important role in the transmission of nerve impulses. The other, the cholinesterase of serum, the so-called "pseudo-cholinesterase", has received less attention because its specific function as an enzyme, as yet, has not been defined. Our own investigations, however, have made use of the latter enzyme as an indicator of albumin metabolism. We have reported(1) a close correlation between human serum cholinesterase and serum albumin levels, confirming the earlier work of Faber(2). In a series of 294 patients with a variety of diseases, we were able to demonstrate in all cases but those with albuminuria, that the serum albumin and the serum cholinesterase levels paralleled each other in a statistically significant manner.

A number of possibilities exist to explain the correlative relationship between the serum albumin and the serum cholinesterase, some of which are: 1) Albumin could act as a precursor to serum cholinesterase, and an albumin deficiency could cause the cholinesterase to decrease, as implied by Kunkel and Ward(3). 2) The decrease could be due primarily to a deficiency in protein intake, resulting in a general decrease of synthesis of all proteins both in serum and in the body organs. 3) The possibility exists that both the albumin and the serum cholinesterase are produced independently of each other, but by similar synthesizing mechanisms.

Methods. Patients were given concentrated normal human serum albumin, intravenously, containing 25 g of protein in 100 cc of neutral buffered diluent. *Protein determinations* were made by the biuret method of Weichselbaum(4) with two modifications: for the precipitation of globulins a solution of 26.8% sodium sulfate was used rather than 23% solution (giving lower albumin values than the customary Howe method which in-

* The albumin used in this study was provided by the National Blood Program of the American Red Cross.

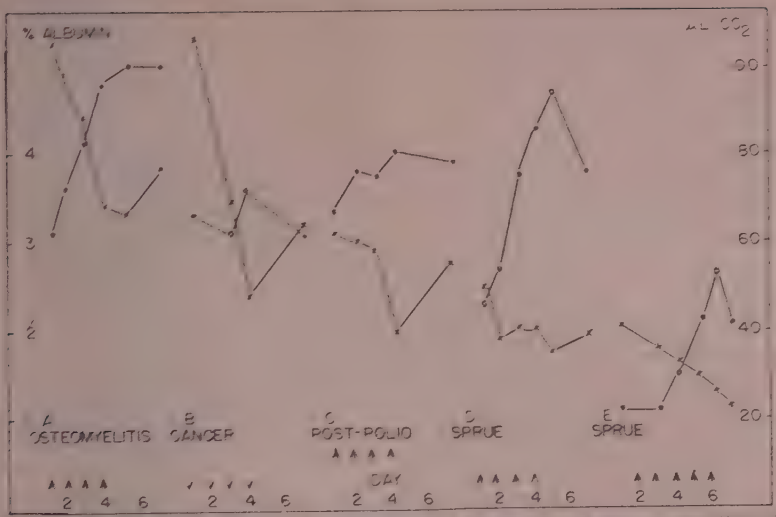

FIG. 1. Initial albumin cholinesterase relationship in five patients without overt liver damage. Arrows indicate administration of 75 g. and V's 25 g. of albumin. Cholinesterase x—x, albumin o—o.

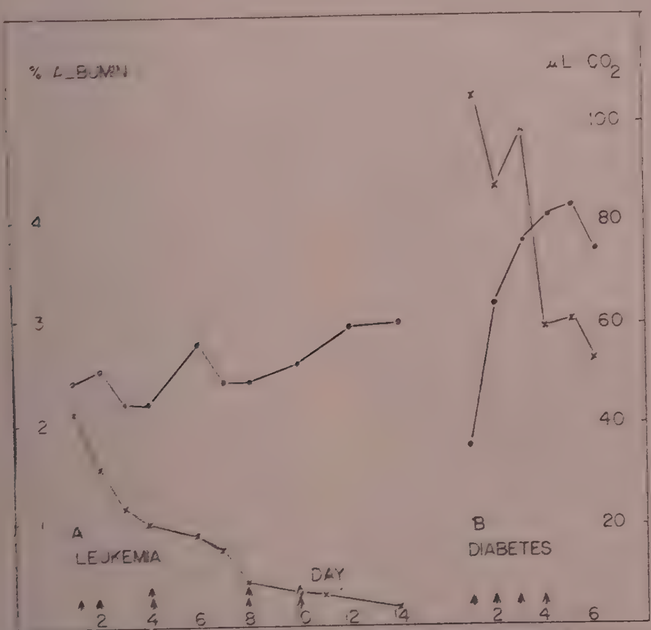

FIG. 2. The albumin cholinesterase relationship in two terminal cases. Arrows indicate administration of 75 g of albumin. Cholinesterase x—x, albumin o—o.

cludes the α-globulin with the albumin); and in spectrophotometric readings of the protein-biuret complex, allowance was made for interference by jaundiced or lipemic sera according to the procedure described by Keyser and Vaughn(5). *Serum cholinesterase activity* was determined by the Warburg technic as previously described by us(6). Results are expressed as μL carbon dioxide at 0°C and 760 mm mercury evolved in a 20-minute

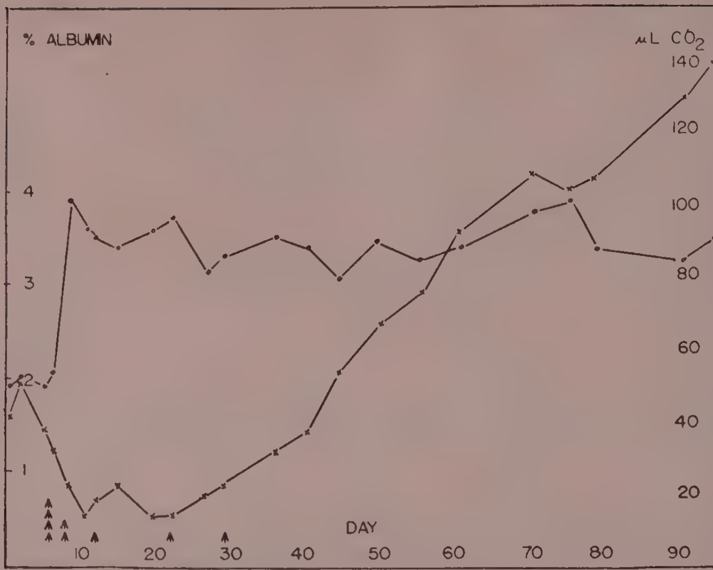

FIG. 3. Patient F. R. The relationship between albumin and cholinesterase in a case of infectious hepatitis. Arrows indicate administration of 75 g albumin. Cholinesterase x–x–x, albumin o–o–o.

period from a buffered bicarbonate solution. All values are corrected for non-enzymic hydrolysis and serum dilution factors. Both protein and cholinesterase values are adjusted to initial hematocrit values as a base line to allow for blood volume changes during albumin administration.

Results. Data are presented in graphic form showing serum albumin and serum cholinesterase values and amounts of administered albumin for a group of patients over varying periods of time. These observations are representative of the general responses observed in a larger series of patients.

Discussion. Of the possibilities suggested above to explain the correlation between the albumin and the serum cholinesterase levels, the first hypothesis, that albumin is a precursor for serum cholinesterase, is contradicted by the fact that, in spite of generally elevated serum albumin levels following intravenous administration of concentrated albumin to patients with initially low serum albumin values, there is neither a simultaneous nor a subsequent increase in the serum cholinesterase (Fig. 1, 2, and 3). Following the administration of concentrated albumin, serum albumin values remain constant or are elevated depending on the relative rate of utilization of administered albumin by the given patient. In most of our cases the administration of the albumin was followed by a rise in serum albumin levels. Despite the administration of from 100 to 300 g of concentrated albumin in these cases, there is a decrease in the serum cholinesterase level, which reaches a minimum on the fourth or fifth day following initiation of albumin administration. This decrease was also noted by Vorhaus(7), incidental to a study on cholinesterase in liver and biliary disease. If albumin were a precursor to serum cholinesterase, we would expect an increase in the cholinesterase values following the intravenous administration of the concentrated albumin; on the other hand, if the production of serum cholinesterase is independent of the amount of albumin present in the serum, we would expect no effect on the serum cholinesterase level following albumin administration. An explanation for the anomalous decrease in serum cholinesterase following albumin administration may be that administered serum albumin depressed the synthesizing systems

which produce both the albumin and the serum cholinesterase.

Possibilities 2 and 3 are not necessarily independent of each other. There is every likelihood that a deficiency of general protein precursors would necessarily restrict all protein synthesis, including albumin and serum cholinesterase. The emphasis in this paper, therefore, is on the third possibility, that albumin and serum cholinesterase are formed by the same or related synthesizing systems and there is good evidence that this synthesis occurs in the liver. The factors which influence albumin synthesis in the liver, whether actual liver dysfunction or a general shortage of protein precursors, cause both the serum albumin and serum cholinesterase values to vary together. Our own experience with patients with liver damage indicated that this enzyme is a valuable tool in the determination of the functional state of the liver.

If it is kept in mind that serum albumin and this enzyme are formed independently, but that the level of the enzyme indicates the ability of the liver to synthesize albumin, it becomes possible to utilize serial determinations of the enzyme as an index of liver albumin synthesis in the presence of administered albumin. Normally, such intravenously administered albumin would interfere with any determination of the ability of the liver to produce this protein by itself. In Patient A (Fig. 2) after intravenous administration of albumin, the albumin levels in the blood remain fairly constant, whereas serum cholinesterase continues to fall as liver failure progresses. Patient B (Fig. 2) is the only patient of the series presented here who shows an unusual initial relationship between the serum cholinesterase and albumin levels. This patient had a history of a long-standing nephritis and albuminuria, and illustrates the only condition in which a normal or high serum cholinesterase level is associated with a very low albumin level. It has been postulated that with albuminuria, the compensatory increase in albumin synthesis simultaneously increases the rate of production of serum cholinesterase(8). Following the administration of serum albumin, the initially high serum cholinesterase value fell. In both of these patients, the final low cholinesterase levels preceded death. In the case shown in Fig. 3, a patient with infectious hepatitis, the administration of albumin was indicated as a therapeutic procedure, but precluded the possibility of using serum protein levels as a guide to improvement. In other liver function tests, such as the cephalin flocculation, the icterus index, and the BSP test, there was no indication as to the course of the disease until the patient was quite obviously improved clinically. The cholinesterase determinations, however, showed that at first severe liver damage was present and the gradual but sustained return of the enzyme level to the normal range preceded obvious clinical recovery. These observations suggest that serum cholinesterase determinations are of prognostic value in the study of liver disease and that they are especially valuable where the administration of albumin intravenously would preclude serial protein determinations.

In summary, the cases presented in Fig. 2 and 3 demonstrate that serum cholinesterase levels vary with changes in the clinical conditions of the patients, independent of changes in serum albumin level as occasioned by intravenous serum albumin administration. This we take as evidence that serum albumin does not act as a precursor for serum cholinesterase. The initial unusual serum cholinesterase to albumin relationship in Fig. 2-B is common to cases of albuminuria. Further evidence that serum albumin is not a precursor for serum cholinesterase is presented in Fig. 1, where despite albumin administration, and following it, rather than an increase in serum cholinesterase levels, there is a depression.

Conclusions. The previously observed(1,2) correlation of serum albumin and serum cholinesterase values is probably due to the simultaneous production of these two proteins by related synthesizing mechanisms in the liver. Albumin does not act as a precursor of serum cholinesterase. Changes in the level of this enzyme reflect the changing ability of the liver to produce both serum cholinesterase and serum albumin. It is suggested that serum cholinesterase determinations are of prognostic value in liver disease and that cholinesterase determinations may be used as

an indication of albumin synthesis, particularly in the presence of administered albumin.

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Adrenal Ascorbic Acid Depletion by ACTH in Nephrectomized and in Subtotally Hepatectomized Rats.* (19487)

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The specificity of the adrenal ascorbic acid depletion test for pituitary adrenocorticotrophic hormone (ACTH) originally devised by Sayers *et al.*(1) is generally accepted, and the method has been widely used in assaying this hormone. In a recent communication it has been reported that when rat ACTH is injected into intact rats, large quantities are found in the kidneys(2). At the same time it cannot be detected in liver or urine(2). Absence from the liver could result from rapid hepatic inactivation. Its presence in the kidneys might reflect the importance of these organs in removing the hormone from general circulation. In view of the rapid disappearance of ACTH from the circulation(3) and the importance of the kidneys and liver in hormonal inactivation in general, it was felt that a study of the effect of these organs upon injected ACTH was warranted. This was tested by determining whether total nephrectomy or subtotal hepatectomy rendered a small quantity of ACTH, in itself sufficient to produce a moderate drop in adrenal ascorbic acid, more effective in this respect.

Materials and methods. Rats of the Holtzman strain were utilized in the present study. In a preliminary assay, designed to suggest a dosage meeting the requirements of the study to be made, a commercial ACTH powder (Armour, standardized against LA-1A) was obtained and from it 4 solutions in physiologic saline containing 4, 2, 1, and 0.5 μg/cc respectively were prepared. Thirteen female rats hypophysectomized 24 hours previously were divided into 4 groups. The left adrenal was removed in each animal under nembutal anesthesia, weighed on a Roller-Smith balance, placed in 6% trichloracetic acid, homogenized and the ascorbic acid determined by the method of Roe and Kuether(4). Each of the 4 concentrations of hormone was administered to a group of animals, each animal receiving 0.5 ml/100 g of the solution to be assayed by tail vein injection. One hour later the animals were sacrificed and the right adrenals re-

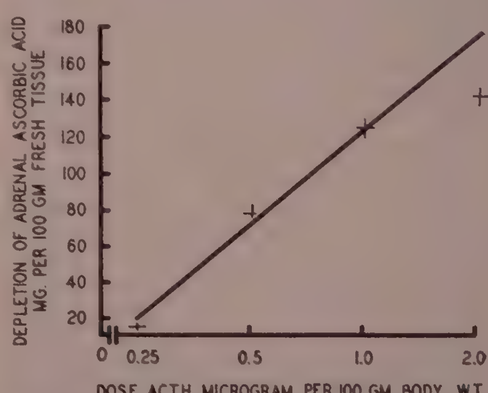

FIG. 1. Adrenal ascorbic acid depletion following intravenous ACTH.

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TABLE I. Effect of Nephrectomy and of Subtotal Hepatectomy on Adrenal Ascorbic Acid Depletion in Hypophysectomized Rats Following Intravenous ACTH.

Group	No. rats	Dose ACTH, $\mu g/100$ g	Ascorbic acid depletion*
Nephrectomized	17	.5	82 ± 11†
Subtotal hepatectomized	16	.5	[illegible]
Control	22	.5	81 ± 8

* Conc. in left adrenal minus conc. in right.
† Mean ± stand. error.

moved, weighed and analyzed for ascorbic acid. The results, summarized graphically in Fig. 1, compared favorably with other results reported for similar preparations(5,6), and a dosage of 0.5 $\mu g/100$ g was selected as fulfilling the requirements of producing a moderate ascorbic acid depletion of 77 mg. Fifty-five female rats were hypophysectomized and divided into 3 groups 24 hours postoperatively. In Group 1, 17 animals were bilaterally nephrectomized and then each received 0.5 μg ACTH in 0.25 ml physiologic saline per 100 g body weight by tail-vein injection. In Group 2, 16 animals each received ACTH in the same amount and manner, following subtotal hepatectomy (70% removed) by the method of Waelsch and Selye(7). Group 3, consisting of 22 animals, received the same hormone treatment without further surgical intervention, and thus served as controls. In each case the left adrenal was removed prior to hormone injection, and in the case of hepatectomized or nephrectomized animals at the time of operation, and the right adrenal extirpated one hour after the injection.

Results and conclusions. The results are summarized in Table I. It is evident that the low dosage of hormone was not rendered more effective by removal of the kidneys. The adrenal ascorbic acid-depletion was identical in animals with and those without kidneys. The inference to be drawn is that the kidneys are not preferentially endowed with the property of inactivating ACTH or withdrawing it from the circulation. Similarly the removal of 70% of the liver was without influence on the ascorbic acid depletion produced by the dosage of ACTH employed. The depletion was actually slightly less in subtotally hepatectomized animals. Although the difference was not statistically significant it invites speculation as to whether total hepatectomy would have produced a more conclusive result.

These experiments, utilizing porcine ACTH, indicate that neither renal nor hepatic tissue is endowed with a prepotent ability to inactivate ACTH or remove it from the circulation.

Summary. Neither total nephrectomy nor subtotal hepatectomy have any effect on the moderate decline in adrenal ascorbic acid which results from the intravenous injection of 0.5 $\mu g/100$ g ACTH. It is concluded that ACTH is not preferentially inactivated or withdrawn from the circulation by either liver or kidneys.

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Plasma Thromboplastin Component (PTC) Deficiency: A New Disease Resembling Hemophilia.* (19488)

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In current concepts of blood coagulation mechanism(1), thromboplastin is said to be liberated from fixed tissue sources through injury, or from blood itself by interaction of a platelet factor and a single inactive plasma factor(2,3). The coagulation defect in hemophilia is thought to be due to absence of the latter plasma factor. We obtained evidence from the study of the coagulation defect of a patient, who had previously been thought to suffer from hemophilia, of the presence of a second plasma factor which appears to be as important biologically as the anti-hemophilic factor for the production of thromboplastin in the blood.

The patient, a 16-year-old Caucasian male with no siblings, living or dead, had a negative family history of hemorrhagic diatheses. Clinical and laboratory findings in infancy suggested a diagnosis of thrombocytopenic purpura, and splenectomy was performed at the age of 2 years. Since then numerous tests of the platelet count and function, bleeding time and clot retraction and of the plasma fibrinogen and prothrombin and serum calcium concentrations have been normal, and venous blood coagulation times have been markedly prolonged. It is not known whether venous blood coagulation time was prolonged prior to splenectomy. The patient has been hospitalized over 100 times for major hemorrhagic episodes, including bleeding from lacerations and hemorrhage into subcutaneous tissues, muscles, joints, gastrointestinal tract, pharynx, and retroperitoneal region. In all instances prolonged coagulation time was markedly shortened and the hemorrhage rapidly controlled by the transfusion of whole blood, fresh or frozen plasma, or freshly lyophilized plasma. The first suspicion that the patient did not have true hemophilia arose when the coagulation time was not significantly shortened by potent preparations of anti-hemophilic globulin (Cohn's fraction I). The prolonged coagulation time is accompanied by a markedly defective prothrombin utilization(4,5), which can be corrected by addition of small quantities of normal plasma *in vivo* or *in vitro*. Further studies have shown that the patient's blood contains normal amounts of all previously described coagulation factors(1), and that abnormal amounts of circulating anticoagulants are not present(6). These findings indicate that the coagulation defect in this patient is due to the absence from his plasma of a previously undescribed coagulation factor. The fact that the defect can be completely corrected with accelerator-free tissue thromboplastin eliminates the possibility of a deficiency in prothrombin or prothrombin conversion accelerators and indicates that the missing factor is concerned with the production of thromboplastin. Accordingly, we have named it the *plasma thromboplastin component* (PTC).

Studies conducted in our laboratory show that PTC can be adsorbed by $BaSO_4$ from oxalated plasma, as are prothrombin(7) and serum prothrombin conversion accelerator (spca)(7,8). It can also be adsorbed by all of previously described prothrombin adsorbants(2) and by Seitz filtration(9). It can be concentrated relatively free of all previously described coagulation factors as follows: Carefully drawn blood is oxalated and centrifuged to remove platelets, using Conley's

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TABLE I. Effect of Various Blood Fractions on Patient's Coagulation Time and Prothrombin Utilization.

Coagulation time,* min	Fraction added (.1 cc added to 2 cc patient's blood)	Coagulation time of mixture, min	Residual serum prothrombin,† %
	Effective fractions		
60	Normal plasma	12	21
120	Supernatant from 20000 r.p.m. plasma (17)	12	25
64	Hemophilic plasma	30	19
60	Berkefeld filtered normal plasma	18	32
60	Hypoprothrombinemic plasma from dicumarol-treated patient	22	40
35	Plasma heated at 56°C 5 min	20	30
36	Normal serum (thrombin free)	11	15
36	Normal serum heated at 56°C 11 min	15	30
60	Euglobulin (9)	5	85
60	.12 mg accelerator free tissue thromboplastin‡	9	20
	Non effective fractions		
70	$BaSO_4$ treated normal plasma	70	80
70	Seitz-filtered plasma	70	85
70	Patient's plasma	70	87
70	Citrate eluate from patient's plasma	70	100
60	.04% to .4% AHG (Cohn's fraction I)	60	90

* Two cc venous blood in 12 × 75 mm glass test tubes at 37°C.

† Modified Ware and Seegers(11). Serum citrated 1 hr after above coagulation time started (Patient's plasma prothrombin is 100%.)

‡ Supplied by Chilcott (CH 61).

technic(10). The "platelet-free" plasma is then heated to 56°C for 5 minutes and the coagulated fibrinogen is removed by centrifugation. The supernatant plasma contains most of the original PTC and anti-hemophilic globulin (AHG)(9) and only traces of prothrombin(11), spca(12,8), Factor V(13), and labile factor(12). The PTC fraction is separated from the AHG by adsorbing it on $BaSO_4$, from which it is eluted with 5.8% sodium citrate.‡ The citrate eluate is then dialyzed against 0.9% NaCl. The final product is free of thrombin. It can be lyophylized without loss of potency. Analysis of one of the products showed that the amount of protein obtained from 1 ml of plasma was equivalent to 8.1 μg of tyrosine(14). The *PTC content* of various plasma or serum fractions can be assayed against the patient's blood in a manner similar to that reported by Graham *et al.*(5) for AHG, using a "2-stage" prothrombin utilization test. The patient's PTC fraction when so assayed is inert, while that obtained from normal, hemophilic, platelet-deficient, or dicumarolized hypoprothrombinemic plasma is significantly active (Table I). PTC concentrate is now under study, using PTC-free "purified" coagulation factors in a system similar to that described by McClaughry and Seegers(15). PTC is associated with the plasma euglobulins obtained either by dilution and acidification of plasma (9) or by 50% saturation of plasma with $(NH_4)_2SO_4$(16). Only traces remain in the other plasma fractions.§ It is most stable in the neutral ranges, being least stable at pH 11.0. Unlike AHG, PTC remains in the serum even after prothrombin, thrombin, and spca have been removed by heating at 56°C for 5 minutes.

PTC is not a platelet factor, AHG, or "complete" thromboplastin, such as that derived from fixed tissue sources, since it will not correct the defect(4) present in "platelet free" normal plasma or in platelet-rich hemophilic plasma. It does not appear to be the plasma

‡ This method of adsorption and elution is similar to Alexander's method(7) for the preparation of prothrombin, except that we start with "platelet-free" heated plasma.

§ Subsequent work has revealed that PTC is precipitated by 50%, but not by 40%, $(NH_4)_2SO_4$ saturation. This further excludes the anti-hemophilic factor which is associated with the proteins precipitated between 0% and 33% $(NH_4)_2SO_4$ saturation(16).

thromboplastin reported by Chargaff and West (17), since the supernatant from plasma centrifuged at 20000 r.p.m. for 150 minutes completely corrects the patient's defect. PTC also is not to be confused with TPC(18), which is Shinowara's term for anti-hemophilic globulin(19). As suggested by Koller(20), the prothrombin conversion factor of Owen and Bollmann, the prothrombin accelerator of MacMillan, the co-thromboplastin of Mann and Hurn, factor VII of Koller, Owren's proconvertin, and spca are probably the same. Since these factors can be adsorbed by $BaSO_4$ and eluted with sodium citrate, it would also seem probable that they contain PTC. A study of this possibility is now in progress. The fact that preparations of PTC do not accelerate a one-stage prothrombin determination, as do the above factors, indicates that our preparation of PTC is free of these factors.

PTC deficiency resembles hemophilia both clinically and in the results of the usual laboratory investigations. It can be distinguished from hemophilia by the following features: 1) the coagulation defect of PTC deficiency cannot be corrected by anti-hemophilic globulin (Cohn's fraction I), Seitz-filtered plasma, or $BaSO_4$-adsorbed plasma; 2) it can be corrected by normal serum, as well as plasma, and by the citrate eluate of $BaSO_4$-adsorbed plasma or serum; 3) hemophilic and PTC-factor deficient bloods correct each other's coagulation defects.

It has been reported that blood from some cases of "hemophilia" is capable of correcting the coagulation defect in other cases of hemophilia *in vitro*(21). This has not been our experience, and it appears more likely to us that such results may be due to mixtures of blood from patients with PTC deficiency and true hemophilia. It is not known whether PTC deficiency is hereditary, and, if hereditary, whether it follows the genetic pattern of hemophilia.

Summary. 1. A severe hemorrhagic disease, characterized by a prolonged whole blood coagulation time due to the delayed formation of thrombin, has been described. The patient with this disease was found to have normal plasma concentrations of all the previously described coagulation factors. 2. The corrective factor in normal plasma or serum can be removed by barium sulfate adsorption. A method for the partial purification and concentration of the patient's missing factor has been outlined. The defect in the patient's blood may be corrected by the addition of plasma free of previously described thromboplastin components (*e.g.* platelet-free hemophilic plasma), as well as by small amounts of tissue thromboplastin. The name *plasma thromboplastin component* (PTC) has been assigned to this previously undescribed coagulation factor.

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Studies on Adrenal Cortex of Pantothenic Acid-Deficient Rat. IV. Adrenal and Serum Cholesterol Levels.* (19489)

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Previous communications from this laboratory(1-4) have dealt with the functional and morphological aspects of the adrenal cortex of the pantothenic acid-deficient animal. The results of these studies have been interpreted as indicative of an adrenal insufficiency in these animals, a conclusion which in certain respects supports the findings of other workers(5,6). In a review of the problem, Cowgill *et al.*(3) discussed certain of the theoretical aspects of the role of this vitamin in adrenal metabolism. One of the possibilities entertained was that coenzyme A (the functional form of pantothenic acid in metabolism) participates in the utilization of acetate in the synthesis of cholesterol, which is recognized as the parent compound of the adrenal corticoids (7,8). It was thus decided to measure the level of cholesterol in the adrenals of such animals. Serum cholesterol and total extractable liver lipids were also determined.

Methods. The animals, diets and conditions were the same as described previously(1). The deficient animals as here referred to indicate those that are placed on the pantothenic acid deficient diet at weaning and allowed to subsist thereon for a period of 5-6 weeks. The theoretical advantages of using an animal of this age have been discussed previously(3). The food intake of control rats (with pantothenic acid) was restricted to approximately that of the deficient animals. Total cholesterol was determined on the adrenals and on serum (from tail blood) by the Sperry-Schoenheimer method(9). Total extractable lipids on the livers were determined by the A.O.A.C. method(10). Since the adrenal cholesterol is known to change in response to "alarming stimuli"(7), animals for adrenal cholesterol determinations were in the "resting state"—*i.e.* care was taken to avoid stressing either group prior to sacrificing them for adrenal cholesterol analysis.

TABLE I. Cholesterol Levels of Adrenal Glands and of Serum of Pantothenic Acid-Deficient and Control Rats.

Group	No. animals	Total cholesterol: Serum, mg %	Total cholesterol: Adrenal, g %
Control	7	92 ± 4	$4.09 \pm .11$
Deficient	10	104 ± 9	$1.36 \pm .21$

All data expressed as mean with S.E.

Differences between the 2 groups with respect to adrenal cholesterol are statistically significant ($p = < .01$). Differences between the levels of serum cholesterol are not statistically significant ($p = > .1$).

Results. Data for the total adrenal cholesterol of 10 deficient and 7 pair-fed controls are shown in Table I. These data show that the deficient animals exhibit a marked depression of adrenal cholesterol, while the control animals are within the accepted range of normal. These findings support our previous demonstration of a lowered adrenal cholesterol by histochemical technics(4). The serum cholesterols of the two groups (Table I), however, do not differ appreciably. Earlier, Scudi and Hamlin(11) had reported that the serum cholesterol of the pantothenic acid deficient dog was reduced. This finding, however, is in all probability related to the "fatty liver" which develops in this species on a pantothenate deficient regimen(11,12). Inanition is also of common occurrence in such animals and this factor undoubtedly exerted some effect on the blood cholesterol. In the rat, under the circumstances of the present experiment, inanition(1) was not a serious problem and no significant differences in total liver lipids was noted between the deficient and control rats. All total lipid liver values were in the normal range of 3-5%.

Discussion. The finding of lowered adrenal cholesterol levels in rats deficient in pantothenic acid may be the result of either: a)

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reduction in synthesis or b) increase in utilization of this steroid. In normal animals, such a finding is usually indicative of recent adrenal cortical stimulation(7). However, in the face of previous evidence pointing to the presence of an adrenal insufficiency of the C-11 oxysteroid hormone in the pantothenate deficient rat(1-4,5,6) it is reasonable to suggest that in these animals the reductions are due to decreased synthesis. In view of recent work indicating that coenzyme A plays a prominent role in acetate metabolism and the finding that acetate can be converted to cholesterol by the isolated adrenal gland(13,14) as well as to the complete hormone(15), this is an especially interesting theory.

Conn *et al.*(16) have adduced indirect evidence that the adrenal cholesterol may arise in part from the serum cholesterol. This finding is interesting in view of the results here presented in which the levels of serum cholesterol are not appreciably reduced. At present, we cannot interpret these conflicting results.

Summary. The 5-6-week pantothenic acid deficient rat shows a marked reduction in the total cholesterol content of the adrenal glands in the "resting state". The serum cholesterols, however, were normal. These animals do not develop fatty livers. It is suggested that in view of previous functional studies on the adrenal cortices of such animals, that the reduction in the cholesterol content of the adrenal glands may represent a decreased synthesis rather than an increased utilization of this steroid for further conversion to hormone. It may be that coenzyme A, the functional form of pantothenic acid in metabolism is a necessary part of the enzymatic complement of the adrenal cortex which synthesizes cholesterol.

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Oncolytic Effect of Egypt Virus on a Human Epidermoid Carcinoma Grown in X-Irradiated Rats.*† (19490)

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It has been reported previously that certain neurotropic viruses possess an oncolytic property for transplantable mouse tumors, and that each of these viruses has its own tumor spectrum, *i.e.* it will destroy or inhibit certain mouse tumors, multiply without damage to the cancer in others, and fail to survive in a few(1). On the basis of these animal experiments, preliminary work has been done on the treatment of human patients with a variety of exotic viruses(2). Unfortunately, no method has existed for determining prior to use on a human patient, whether the virus employed has any oncolytic ability for the specific human tumor against which it is to be directed. The recent finding, however, that human tumors as well as normal tissues could be grown in x-irradiated heterologous hosts (3) especially in the rat, which, in contrast to mice, is immune to most of the viruses used, has made available what is hoped will be such a screening tool. Report will hereby be made of a human tumor against which Egypt 101 virus showed oncolytic ability both in x-irradiated rats and in tissue culture. Even though it has not been possible, to date, to test the virus in the original tumor donor, since he has not shown recurrence of his disease after operation, it is felt that both the method and the oncolysis are noteworthy.

Materials and methods. The tumor used, obtained immediately after surgical removal, was a metastatic epidermoid carcinoma from the jaw of a 75-year-old male, "C.K.", who had had a primary carcinoma of the lip removed 18 months previously. As soon as received, the tumor, handled with aseptic technic, was minced fine enough with scalpels so that it would pass through a No. 18 needle when suspended in a buffered Ringer's solution. 0.5 cc of this suspension was implanted subcutaneously in each flank of 16 weanling female rats (Carworth Farms) that had received 2 total body doses of x-irradiation, 150 r each, on consecutive days, 3 days prior to the implantation. Seven days after implantation, microscopic examination of the tumors of one of the rats showed "healthy", well vascularized growths, full of mitotic figures. As previous experiences had indicated that the human tumors either grew or failed uniformly in all the animals implanted, it was presumed that the "K" tumor was suitable for virus study. Accordingly 9 days after implantation 9 of the 15 remaining animals were injected directly into one tumor only with 0.1 cc of Egypt 101‡ virus as a 40% stock mouse brain emulsion, an inoculum which subsequently titered 10^{-7} intracerebrally in mice. This virus was chosen because it had been used clinically with no ill effects on patients and because prior tests had also shown that the rat whether normal or irradiated was insusceptible to it. One virus treated animal was killed daily, following inoculation, for 7 days (except that 3 were killed the 1st day). A cross section of each tumor was taken for histological examination and the remaining tumor after grinding, was made up to a 10% suspension in horse serum saline and titered for virus, intracerebrally, in mice. Control animals were killed on the 1st, 3rd, 5th (3 rats) and 7th days after the inoculation. Tumor material obtained from the 3 control rats killed 14 days after implantation was used in several ways: it was transferred successfully to a 2nd generation of x-irradiated

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‡ Obtained through the courtesy of Dr. John Paul of Yale University; it is immunologically identical with West Nile virus.

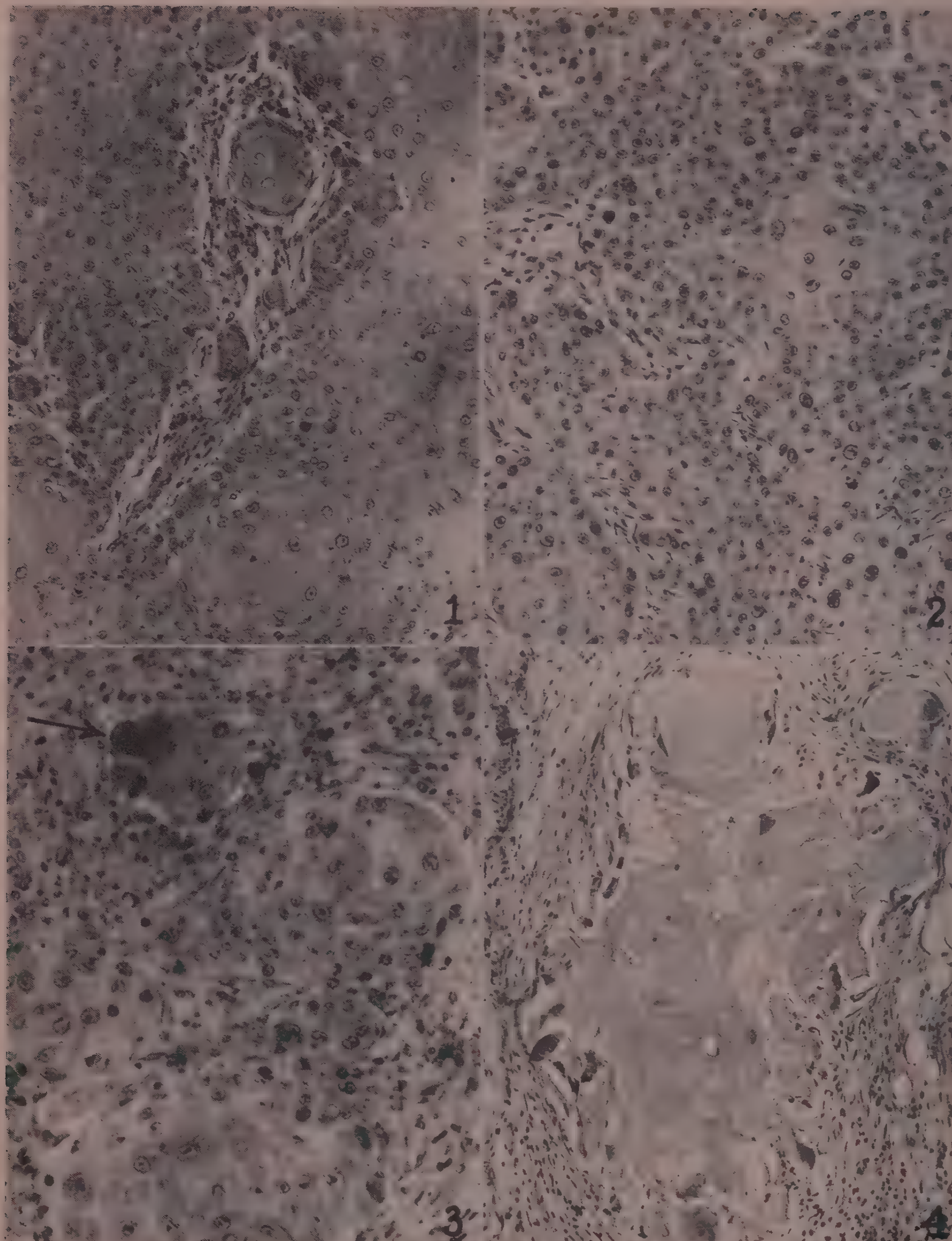

FIG. 1. Metastatic epidermoid carcinoma from neck of patient "K"; original tumor. × 120.

FIG. 2. Same "K" tumor after 11 days in an x-irradiated rat; virus injected 2 days previously. × 120.

FIG. 3. "K" tumor after 13 days in an x-irradiated rat; virus injected 4 days previously. Note degenerating cells (arrow). × 200.

FIG. 4. "K" tumor after 15 days in an x-irradiated rat; virus injected 6 days previously. × 120.

FIG. 5. Control "K" tumor grown 14 days in x-irradiated rat. × 80.

rats in which it grew well; it was grown on the chorioallantoic membrane of the chick where it exhibited a high degree of mitotic activity; and it was placed in roller tube tissue culture, using a horse serum-placental extract-Gey's solution mixture as nutrient. Each of the 16 plantings (2 to a cover slip) in the tissue culture showed good and remarkably uniform outgrowth of tumor 7 days after planting. On this day in order to test the effect of the virus on the tumor cells *in vitro* culture, as well as in the rat, the 8 cover slips with growing tumor were placed, 2 each, in 4 tubes. In 2 of the tubes the feeding material was mixed with the same Egypt 101 stock mouse brain emulsion used in the rats, in such manner that the final titer of virus in the tubes was 10^{-3}. To the feeding mixture in the 2 control tubes, normal mouse brain emulsion was added in amount equal to that in the virus treated tubes. The final volume of fluid material present in each tube was 1 cc. For the first 3 days, 0.1 cc was removed daily from each of the 2 tubes containing virus, the material pooled and titered in mice. A similar amount was withdrawn from the control tubes. Replacement was made of any amount removed by an equal quantity of ordinary tissue culture feeding solution. After the first 3 days, *i.e.* on the 4th, 7th, 9th and 10th days, 0.2 cc was taken from each tube and separate titrations done. Ten days after the original addition of virus (17 days after planting) all the cover slips were fixed and stained for microscopic examination. By this time it was estimated that all the original fluid in the tubes had been replaced by fresh extract.

Observations and discussion. All the tumors grown in untreated control rats were found to be in excellent condition, identical morphologically to the original carcinoma (Fig. 1), well vascularized and full of mitotic figures. Those removed on the 14th day after implantation (3 rats) were especially large, solid and free from necrosis (Fig. 5). The tumors removed from treated rats (4 animals) in the first 2 days after injection with virus were also growing well (Fig. 2); while those removed on the 3rd, 5th and 6th days were entirely dead (Fig. 4) and those of the 4th day degenerating (Fig. 3). The tumors of the 7th day had a peculiar appearance in that large central areas of the tumor were dead but foci about these regions remained alive. It

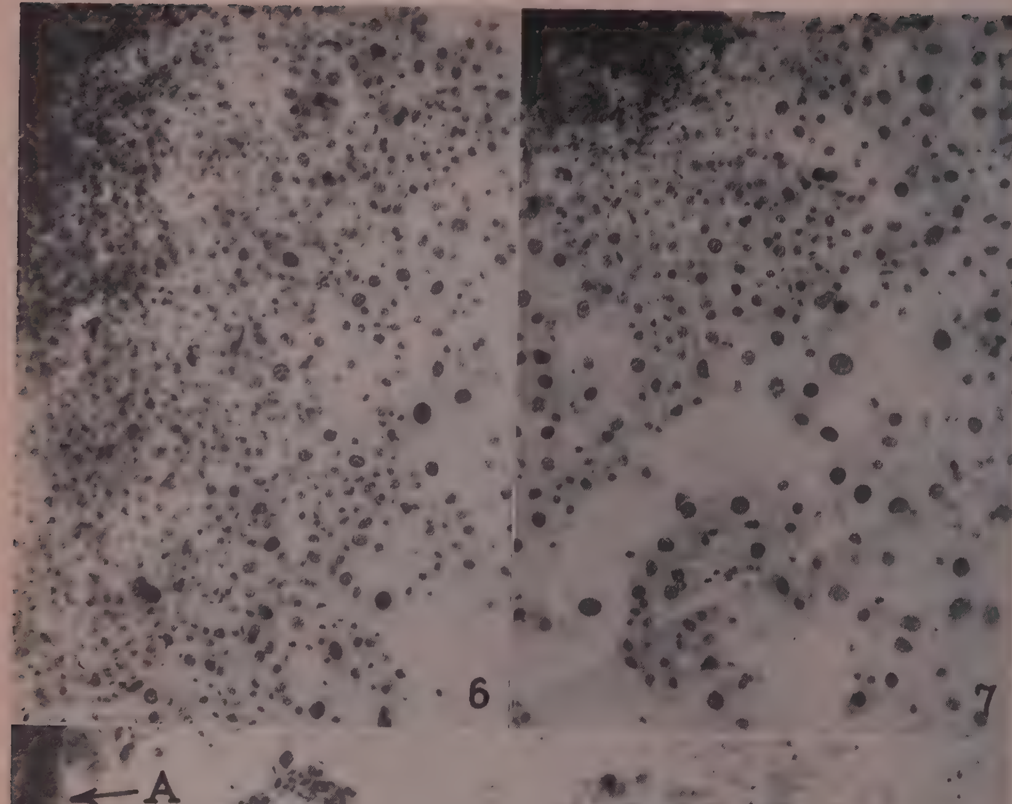

FIG. 6 and 7. Two control tissue cultures of "K" tumor, first grown 14 days in an x-irradiated rat (Fig. 5) and then 17 days in tissue culture. × 80.

FIG. 8 and 9. Two virus treated tissue cultures of "K" tumor, same age (17 days) and from same rat as controls (Fig. 5 and 6); Egypt 101 virus added 10 days previously after the tissue cultures had grown 7 days normally. Tumor cells have retracted toward original planting (arrows "A") or lysed (arrow "B"). × 80.

TABLE I. Titer of Egypt 101 Virus in Tissue Cultures of "K" Tumor.*

Day after virus added to cultures	Virus titers† Tube 1	Tubes 1 & 2 (pooled)	Tube 3
1		$10^{-5.25}$	
2		$10^{-5.50}$	
3		$10^{-6.25}$	
4	$10^{-6.25}$		$10^{-5.25}$
7	$10^{-4.25}$		$10^{-5.50}$
9	$10^{-3.75}$		10^{-4}
10	10^{-5}		10^{-5}

* Original titer of virus in tissue culture = 10^{-8}. Virus added to cultures, 7 days after planting.

† Titers determined by intracerebral inoculation of test fluid into Swiss albino mice. See text.

should be noted that though the virus had been injected into the tumor of one flank only, both tumors of any one animal were comparable. (The injected tumors were found to titer, in mice, approximately $10^{-3.5}$ except that the 5th and 6th days were 10^{-1} and the 7th $10^{-2.5}$. The uninjected tumors had no titer for the first 2 days, 10^{-1} on the 3rd and 6th days, and 10^{-2} on the 4th and 7th days.)

Microscopic examination of the tissue cultures of "K" tumor showed an even more striking contrast between the control and virus treated tumors. Each of the 8 explants in the control tubes was still in luxuriant condition (Fig. 6 and 7) 17 days after planting, while every one of the 8 treated explants showed complete lysis of the tumor cells or retraction toward the original site of outgrowth (Fig. 8 and 9). (Since the human carcinoma possessed very little rat stroma at the time of planting and had been almost "pure" tumor (Fig. 5) very few normal rat cells had grown out. It is noteworthy that the few fibroblasts and macrophages present appeared undamaged even in the virus treated tubes.) The virus titrations of the tissue culture fluid (Table I) showed that considerable multiplication of virus occurred. By the 10th day all the original volume of virus containing feeding fluid had been replaced by ordinary nutrient, yet the titers remained very high. It would have been interesting to see how long these titers would have persisted after complete lysis of the tumor cells. It is possible that the normal rat cells present accounted for some of the titer, yet they were so few in number that the amount must have been very small indeed.

It thus seems apparent that Egypt 101 possessed oncolytic activity against the epidermoid carcinoma of "C.K." whether grown *in vivo* in a complex host such as a rat, or *in vitro* in tissue culture. The final test, of course, would be to treat "C.K." with Egypt virus if he has a recurrence of his tumor.

It is hoped that this method of testing the oncolytic activity of a virus against any particular tumor will be of considerable practical value and give a more rational approach to therapy with oncolytic viruses. The work with viruses on transplantable mouse tumors has indicated a very specific tumor spectrum for the oncolytic activity of each virus used. Unpublished data of ours indicates that this finding will hold true for the human tumors as well since it has been found that in 2 cases (a recurrent mixed tumor of the parotid and a metastatic squamous carcinoma), Egypt 101 reproduced in the tumors grown in rats but caused no apparent damage. These tumors were transferred after 14 days into 2nd generations of x-irradiated rats and still had titrable virus (10^{-3}) when the 2nd groups of animals were killed (28 days after surgical removal).

Since Moore(4) has indicated that the oncolytic activity of a virus can be increased by passage in mouse tumors it is also possible that a similar procedure and result can be carried out by continued virus passage in human tumors borne by the x-irradiated rats. Work is now under way to test this.

Summary. 1. Egypt 101 virus was found to possess oncolytic activity against a human epidermoid carcinoma grown either in x-irradiated rats or in tissue culture. 2. It is suggested that this rat-tissue culture method of testing the oncolytic activity of a virus for any particular tumor may be employed in estimating the possible value of virus therapy for each individual patient.

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Failure of Rutin and Related Flavonoids to Influence Mortality Following Acute Whole Body X-Irradiation.* (19491)

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The flavonoids were the first compounds reported to reduce the mortality in animals following whole body x-irradiation. The rationale for the use of the flavonoids in the attempt to prevent or treat radiation injury has been based upon the claim that these compounds reduce the hemorrhage resulting from increased capillary fragility(1). Rekers and Field(2) observed a reduced hemorrhagic tendency and obtained improved survival rates among irradiated dogs pretreated with rutin or other flavonoids. Clark, Uncapher, and Jordon(3) obtained increased survival of guinea pigs treated with calcium flavonate. Sokoloff, Redd, and Dutcher(4) found that CVP, a citrus "vitamin P" compound, significantly reduced the mortality of rats exposed to 800 r of x-radiation. Opposed to these findings are the negative results of other workers. Kaplan(5) and Cronkite *et al.*(6) used rutin in mice, and Kohn and coworkers (7), and Patt and his associates(8) used rutin in rats without observing any effect on radiation mortality. Similarly CVP had no beneficial effect in mice(9), and rutin and hesperidin failed to influence the mortality or hemorrhagic changes in irradiated young chicks(10).

In view of the conflicting reports regarding the value of the flavonoids in reducing the mortality of animals exposed to acute whole body x-radiation, the present investigation was undertaken in order to explore further the possible usefulness of these compounds.

Materials and methods. The flavonoids used include: rutin in pure form, rutin in de-ionized form, rutin solubilized with piperazine hexahydrate, quercitrin, quercetin, hesperidin, dihydroquercetin, naringen, calcium flavonate, hesperidin methyl chalcone, xanthorhamnin, hemoeriodictyol, morin (de-ionized), citrus vit. P (CVP), and lemon juice infusion (LPI). CVP is reported(9) to contain 4% of a quercitrin-like substance, 15% eriodictin, 80% hesperidin-glucose, and 0.38% calcium phosphorus ash. Lemon peel infusion contains eriodictyol glycoside, hesperidin, perhaps other flavonone glycosides, i-inositol, some fractions of the vit. B complex, and some natural soluble carbohydrates. A total of 1132 CF_1 female mice and 232 female Sprague-Dawley rats were used. The mice were 7 to 9 weeks in age and weighed 18 to 24 g prior to irradiation. The rats were 10 to 18 weeks old and weighed 116 to 246 g at the time they were started on the flavonoid treatment. In addition, 70 female guinea pigs with a weight range of 205 to 400 g were included in the studies. The mice and rats were fed Wayne Dog Blox. The guinea pigs were given Purina Rabbit Chow Meal supplemented daily by fresh cabbage and timothy hay. Animal weights, deaths, and food and drinking water consumed, if containing a flavonoid, were recorded daily. In all tests the administration of the compounds was con-

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tinued post-irradiation until there were no survivors or until the 30th day post-irradiation.

Rutin was solubilized by the addition of an aqueous solution of piperazine hexahydrate to a suspension of rutin in water. To solubilize 25 mg of rutin in 1 ml of water, 20 mg of piperazine were required. The pH of the resulting solution was 9.1-9.3. Clark and MacKay(11) have reported that in the body rutin splits off from the piperazine hexahydrate. The other insoluble flavonoids were dissolved in propylene glycol, while the soluble flavonoids were dissolved in water. An x-ray unit operated at 250 kvp and 15 ma was used for all exposures. Inherent filtration of the tube was 0.25 mm Cu, and a filter of 0.25 mm Cu + 1.0 mm Al was added. The radiation intensity was measured with a Victoreen condenser r-meter corrected for temperature and pressure. For the mice the target distance was 60 cm and the dose rate 48-50 r/min.; for the rats and guinea pigs the distance was 75 cm and the dose rate 31-36 r/min. The animals were exposed to a revolving cage divided into concentrically arranged compartments. Each flavonoid-treated group of animals, except where specified, was irradiated simultaneously with an equal number of untreated control animals. The effect of each treatment itself was tested in groups of unirradiated animals, with normal (untreated and unirradiated) animals serving as controls.

Results. Table I shows the schedule of flavonoid treatment and the x-ray doses received by each group of animals, along with the 30-day mortality results obtained.

Rutin administered by various methods in mice. As demonstrated by the data in the table the 30-day mortality among irradiated mice was not diminished by treatment with rutin given by intraperitoneal injection, by single daily administration by stomach tube, or by incorporating it in the drinking water or in the food. Furthermore, no significant difference was noted between the treated and untreated animals with respect to weight loss or time of death after irradiation. Of these rutin treatments the only one which adversely affected the control unirradiated mice was the 5% rutin diet. This caused a 4.3% weight loss of 12 mice which consumed it for 7 days. For this reason the group of mice receiving this diet before being irradiated was placed on the 2.5% rutin diet following irradiation.

Other flavonoids orally or in diet of mice. To determine whether other flavonoids might provide some protection different compounds were given orally or in the diet according to the schedules outlined in the table. All the mice thus treated appeared healthy and active and maintained a body weight equal to that of the untreated controls. In these tests none of the flavonoids exerted any favorable influence on 30-day mortality, on time of onset of morbidity or mortality, or on weight loss.

Comparison of the 30-day LD_{50} of x-ray in rutin treated and untreated mice. The mortality data for all rutin treated mice exposed to 700, 600 and 500 r were pooled together and compared with those of the untreated irradiated controls. This involved a total of 224 mice treated with the various forms of rutin by different routes of administration and 294 mice which served as untreated irradiated controls. When the percent mortality for each group at 700, 600, and 500 r was plotted by the log-probit method the 30-day LD_{50} was estimated to be 550 r for the rutin treated animals and 560 r for the controls. The close approximation of the slopes of the two curves was a further indication that the rutin had no influence on the lethal effects of whole body x-irradiation at the x-ray doses used.

Flavonoids in rats. Solubilized rutin, hesperidin methyl chalcone, and CVP were administered to rats through their drinking water according to the tabulated schedule. Half of each group of 18-week-old rats (see table) was given daily for 7 days post-irradiation, via stomach tube, 40 mg of flavonoid in 2 ml of water in addition to the flavonoid they were taking in the drinking water. This treatment was supplemented by daily subcutaneous injections of 100 mg of sodium ascorbate in 0.5 ml of distilled water during the 7-day post-irradiation period. Two groups of younger rats, 11 and 10 weeks old, respectively, received no treatment other than CVP in the drinking water. The results of these

tests showed no evidence of protection against injury by x-rays in rats.

Flavonoids in guinea pigs. Seventy young female guinea pigs were divided into 7 groups

TABLE I. Mortalities Among Various Species of X-irradiated Animals Untreated and Treated with Flavonoid Compounds.

Flavonoid	Procedure	Species and No. of animals Control	Treated	Dose whole body X-ray (r)	% mortality, 30 days Untreated	Treated
		CF_1 mice				
Solubilized rutin	125 mg kg intraper. daily 7 days pre-irrad. and continued post-irrad.	12	12	800	100	100
		12	12	700	91.7	100
		12	12	600	83.4	75
		12	12	500	25	25
Solubilized rutin	Orally by stomach tube 5 mg in .2 ml H_2O daily 7 days pre-irrad. and continued post-irrad.	12	11	800	100	100
		11	10	700	100	100
		10	12	600	80	83.4
		12	12	500	41.6	33.4
Solubilized rutin	.1% in drinking water 14 days pre-irrad. and continued post-irrad.; mean daily intake 5 mg per mouse.	24	24	800	100	100
		24	24	700	95.8	100
		24	24	600	85.4	79.2
		24	24	500	25	29.2
Powdered rutin in pure form	5%, 2.5% or 1% in food for 7 days pre-irrad. and continued post-irrad. at conc. of 2.5% or 1%					
	Mean daily doses					
	5 % 177.4 mg	12	12	700	100	100
	2.5 113.2	12	12	700	91.7	83.4
	1 42.7	12	12	700	100	100
	2.5 126.8	12	12	600	58.4	66.7
	1 39.2	12	12	600	75	75
Hesperidin methyl chalcone in H_2O	Orally: 7 days pre-irrad. and continued post-irrad. Daily doses, 10 mg in .2 ml		12	700	*	100
Citrus vit. P (CVP) in H_2O			12	700	*	91.7
Xanthorhamnin in H_2O			12	700	*	91.7
Dihydroquercetin in PG	(PG=propylene glycol)		10	700	*	100
Morin (de-ionized) in PG			10	700	*	100
Homoeriodictyol in PG			12	700	*	100
Rutin (pure) in PG			11	700	*	100
Rutin (de-ionized) in PG			11	700	*	100
	2.5% in diet for 14 days pre-irrad. and continued post-irrad. Mean daily dose, mg:					
Quercitrin	89.5		12	700	†	100
	101		12	600	‡	66.7
Quercetin	166		12	700	†	100
	111.5		12	600	‡	75
Dihydroquercetin	112.2		12	700	†	100
	100		11	600	‡	63.6
Naringen	120.2		12	700	†	91.7
	195		12	600	‡	66.7
Calcium flavonate	94		12	700	†	100
	207		10	600	‡	60
Hesperidin	105.2		12	700	†	83.4
	101		12	600	‡	66.7
Hesperidin methyl chalcone	92.2		12	700	†	91.7
	104		12	600	‡	75
Citrus vit. P (CVP)	180		12	700	†	100
	201		12	600	‡	75
Lemon peel infusion (LPI)	123.2		12	700	†	100
	111.5		10	600	‡	70

TABLE I. (Continued.)

Flavonoid	Procedure	Species and No. of animals Control	Treated	Dose whole body X-ray (r)	% mortality, 30 days Untreated	Treated
		Rats 18 wk old				
Solubilized rutin	.1% in drinking water 21 days pre-irrad., plus 100 mg of sodium ascorbate subcut., and 40 mg of flavonoid by stomach tube daily post-irrad.	22	22	700	100	90.9
Hesperidin methyl chalcone		24	24	700	100	100
Citrus vit. P (CVP)		24	24	700	83.3	100
		Rats 11 wk old				
CVP 32.2 mg daily	14 days pre-irrad. in drinking water, and continued post-irrad.	26	26	600	46.1	68.4
		Rats 10 wk old				
" 29.3 " "		20	20	500	45	40
Mean daily doses (mg)		Guinea pigs				
Solubilized rutin 73.5	.1% in drinking water 21 days pre-irrad. and continued post-irrad. supplemented with subcut. inj. of 100 mg sodium ascorbate daily post-irrad. for 7 days		10	250	§	50
Solubilized rutin 76.2 + vit. C 100			10	250	§	70
CVP 82.3			10	250	§	90
Hesperidin methyl chalcone 84.1			10	250	§	100

* Pooled 30-day mortality of 93 untreated mice at 700 r = 94.3%.
† " " " " 119 " " at 700 r = 97.5%.
‡ " " " " 118 " " at 600 r = 66.9%.
§ " " " " 30 " guinea pigs at 250 r = 43.4%.
Mean daily doses in food and drinking water were calculated on basis of pre-irradiation intake.

of 10 each; 40 were given flavonoids in the drinking water and 30 served as controls. Sodium ascorbate was added daily to the drinking water of one of the two groups receiving the solubilized rutin. Each animal in this group received approximately 100 mg of vitamin C per day. These flavonoids, with or without vit. C, did not provide any protection against the lethal effects of x-ray nor against the weight loss of those animals which survived for 30 days. Indeed, the mortality and weight losses shown by the treated animals were consistently greater than those shown by the untreated ones.

Discussion. The most prominent clinical manifestation of exposure to x-radiation is described by Warren and Draeger(12) as the hemorrhagic tendency reflected in moderately severe cases by hemorrhagic gingivitis, petechiae, epistaxis, hematemesis, and bloody diarrhea. These changes have also been described in animals(13). Thus it was logical for investigators(2-4) to administer rutin and related flavonoids in the hope that the prevention of such effects of ionizing radiation might favorably affect mortality in animals. Rutin had become the flavonoid of choice for the control of bleeding believed due to increased capillary fragility. However, any agent effective in offsetting the bleeding tendency induced by x-irradiation would be expected to influence mortality only to the extent to which the hemorrhagic syndrome contributes to death. The possibility that bleeding may play a more important role in some species than in others in causing radiation death should perhaps not be neglected in the attempt to explain the discordant results of various investigators. It is of interest to note that in the findings reported in the literature on the influence of the flavonoids on radiation mortality in animals there are no direct contradictions. Of the 3 important experimental variables, namely animal species, specific flavonoid tested, and route of administration, no 2 groups of workers used the same combination. Those reporting positive results used some manner of oral administration of a flavonoid agent in dogs(2), guinea pigs(3), and rats(4), while those describing negative findings used a parenteral route in mice(6,9), rats(7,8), and chicks(10), or the oral route in mice(5). In 2 of these studies, one negative(7), and the other positive(4), rats were treated orally in

both, but the flavonoids tested were different.

The results of the present study introduce only one questionable direct contradiction to the work of others who report a protective action of a flavonoid against the lethal effect of radiation. Negative results were obtained by us when rats were treated orally with CVP, contrary to the results obtained by Sokoloff *et al.*(4) under the same general conditions. These workers, however, used a British brown breed of rat treated by repeated single oral doses of the flavonoid, while we used Sprague-Dawley rats treated by the CVP in the drinking water. The total daily dose of CVP administered was much larger in our tests and the pre-irradiation treatment period was longer. In the present studies on mice, rats and guinea pigs, there was no evidence that the flavonoids offer any protection to these species against the lethal effects of acute whole body x-irradiation. These results provide general confirmation of the negative findings of others(5-10) in this regard. More direct confirmation of the negative findings reported by others is seen in the results of the present tests in which rutin was given intraperitoneally to mice, simulating the conditions of Cronkite *et al.*(6), and in which rutin was given to mice in the drinking water or food, simulating the conditions of Kaplan(5).

The data of others have shown that the flavonoids have a very low toxicity(14,15). The parenteral injections of 50 mg/kg of rutin in rats and guinea pigs, and of 200 mg/kg in rabbits was harmless(14). In the present study, mice tolerated a daily intake of approximately 250 mg/kg of solubilized rutin in drinking water for 30 days. The daily oral administration, for 21 days, of rutin and the related flavonoids in doses as high as 500 mg/kg in distilled water or in propylene glycol was not harmful. Likewise, mice given 2.5% flavonoids in the diet for 14 days showed no significant weight losses. Intraperitoneal injection of 125 mg/kg of rutin for 7 days in mice, and large amounts of the soluble flavonoids orally in rats had no adverse effects. The possibility may exist, however, that the amounts of CVP and hesperidin methyl chalcone consumed by the guinea pigs had a deleterious effect since the mortality and weight loss in these tests was significantly greater in the treated than in the untreated animals. In this case, however, the supplemental vit. C should not be disregarded as a possible contributing factor.

The fact that workers reporting positive results in testing flavonoids against radiation lethality(2-4) all administered the compounds by the oral route is of interest in view of the studies of Clark and MacKay(11) on the metabolism of a number of flavonoids when given orally. These workers found in human experiments that urinary excretion was negligible following large oral doses (50 mg/kg) and that rutin is not recoverable from the stools and is destroyed when incubated in an aqueous stool suspension. In animals, the percentage of the oral doses excreted in the urine was negligible. Some of the fed compounds were almost completely recoverable from the gastrointestinal tract. They reported that the more soluble flavonoids were no better absorbed than the more insoluble ones such as rutin. The insoluble complexes of rutin dissociated to the typically insoluble rutin in the intestine. The work of Clark and MacKay(11) supports the cumulative evidence that it is unlikely that the flavonoids are vitamin-like and that they exert any significant physiologic effect.

Various authors(16-18) have suggested the possible value of the flavonoids under conditions of stress in which there is an increased demand for ascorbic acid. Allen(19) suggested that rutin and ascorbic acid together might be more effective in reducing the hemorrhagic state in the radiation syndrome. In the present study, however, this combination in guinea pigs, which are unable to synthesize vit. C, and in rats gave no indication of a beneficial effect on radiation mortality.

Summary. 1. Rutin, rutin complexed with piperazine hexahydrate, and de-ionized rutin administered to mice by different routes had no protective action against the lethal effects of whole body x-irradiation in doses of 500, 600, 700 or 800 r. 2. Quercitrin, quercetin, hesperidin, hesperidin methyl chalcone, dihydroquercetin, naringen, calcium flavonate, xanthorhamnin, morin (de-ionized), citrus vit. P (CVP), and lemon juice infusion (LPI),

administered in the diet, had no protective action on mice exposed to acute whole body x-radiation. 3. Rutin complex, hesperidin methyl chalcone, and citrus vit. P had no protective effect on rats and guinea pigs exposed to acute whole body x-irradiation. Supplemental vit. C did not enhance the action of the flavonoids or decrease mortality. 4. The LD_{50} of 250 kvp x-ray, based on the 30-day mortality, for the CF_1 mice used in this experiment, was approximately 550 r.

Rutin, de-ionized rutin, de-ionized morin, dihydroquercetin, homoeriodictyol and xanthorhamnin were supplied by Dr. S. H. Wender and Dr. T. B. Gage of the University of Oklahoma; rutin, quercitrin and quercetin by Dr. J. F. Couch of the United States Department of Agriculture; solubilized rutin in sterile ampules, rutin and piperazine hexahydrate by the Abbott Laboratories; hesperidin, hesperidin methyl chalcone, naringen, calcium flavonate, and lemon juice infusion (LPI) by Dr. A. J. Lorenz of the California Fruit Growers Exchange; and citrus vitamin P (CVP) by the Vitamerican Oil Co.

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Eosinopenia Produced by ACTH in Patients with Schizophrenia. (19492)

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The contention that adrenal cortical function is deranged in patients with schizophrenia, particularly those with long-standing disease, has been advanced for several years by Pincus, Hoagland, and their associates(1). Using changes in urinary excretion of sodium, potassium, uric acid, and steroids, and in the level of circulating blood lymphocytes as indices, they have reported that as few as 20% of schizophrenic subjects respond in normal fashion to the injection of 25 mg of pituitary adrenocorticotropic hormone. Despite the

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TABLE I. Fasting Eosinophil Levels and Fall After ACTH in Group of Schizophrenic Patients.

	Nov., [illegible]		Dec., [illegible]	
Subject	Fasting eosinophils, mm³	% fall 4 hr after ACTH, 25 mg i.m.	Fasting eosinophils, mm³	% fall 4 hr after ACTH, 25 mg i.m.
B	273	68	283	67
E	275	48	250	29
Me	53	43	53	62
N	153	69	127	38
S	211	48	294	52
T	12	42	122	52
G	83	82	138	43
H	83	66	80	69
K	381	49	379	47
L	75	64	64	*
Mi	155	51	148	29
R	200	24	172	15
Sr	46	59	52	61
F	103	65	146	*
Po	72	63	77	90
Ha	177	72	356	72
J	267	36	439	53
Mic	664	63	711	59
Ni	67	69	57	*
LW	202	78	155	74
A	323	48	139	88
Ma	314	63	459	*
P	43	58	77	61
Ra	226	50	208	*
Si	127	70	103	85
Su	250	18	242	33
JW	127	32	472	44
Kr	253	71	200	*
Ku	283	65	*	*
Mc	111	50	*	*

* Not determined.

demonstration by other investigators of an apparently normal response to ACTH by patients with schizophrenia(2-4), the postulated hypoadrenocorticism in such patients has been used as a rationale for therapy of this disorder with cortisone(5). In addition, the schizophrenic subject's response to ACTH has been considered of prognostic significance in terms of the anticipated efficacy of electroconvulsive therapy(6). Hence it seemed pertinent to present material on the eosinopenia produced by ACTH‡ in 30 patients with long-standing mental illness.

‡ Supplied by Armour & Co., Chicago, Ill.

Subjects and method. These data have been accumulated during the course of a project now in progress at the Elgin State Hospital designed to investigate nicotinic acid-tryptophane relationships in human subjects. The group being studied includes 30 male patients, aged 30 to 63 years, who have been hospitalized because of schizophrenia for 2 to 19 (average: 11) years. Eosinophil counts were determined on venous blood before, and 4 hours following intramuscular injections of 25 mg of ACTH, according to the method of Thorn(7). The initial studies were carried out prior to the institution of the experimental regimen, and the procedure was repeated after the subjects had been on a moderately low-protein diet for one year.

Results. All but 5 patients showed on at least one occasion a 50% or greater fall in circulating eosinophils following the injection of ACTH (Table I).

These observations are consistent with a normal eosinopenic response to ACTH in at least 25 of 30 patients with schizophrenia of long duration. Also of interest is the close correlation in several subjects of the 2 fasting eosinophil levels determined one year apart.

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Alterations in Circulating Platelets Following Administration of Adrenocorticotropic Hormone. (19493)

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Since adrenocorticotropic hormone (ACTH) has become available for clinical use its effect upon the hematopoietic system has been studied. The small amount of information which is available with regard to the behaviour of circulating platelets after ACTH administration has been conflicting. Zondek and Kaatz(9) studied the effect of thyroid extract and adrenocortical extract on platelet levels. They noted a decrease in circulating platelets in a number of patients 2 hours after the injection of Cortigen (cortical extract Richter). Adams(1) noted no change in platelet levels after administering adrenal cortex extract to normal rats. Monto, Brennan, Margulis and Smith(5) studied the effect of ACTH and cortisone in blood coagulation and noted no significant platelet changes. Koller and Zollikofer(4) found platelet levels in 4 patients moderately increased after administration of small doses of ACTH. This increase was preceded in two of the cases by a transitory decrease. Jacobson and Sohier(3) have noted an increase in circulating platelets following administration of ACTH in idiopathic thrombocytopenic purpura and refer to others with similar experience with this disease.

Method. Circulating platelets were counted in normal individuals at regular hourly or 2 hourly intervals following the injection of ACTH. Twenty essentially healthy individuals, one of whom had previously undergone splenectomy, were studied. None were febrile, cachectic or suffering from myeloid, hepatic or splenic disease. Eosinophil counts were taken simultaneously in all instances. Blood was drawn from an arm vein with clean dry syringes and needles and 4 cc samples were transferred to small dry bottles containing 2.0 mg potassium oxalate and 3.0 mg ammonium oxalate. The bottles were agitated gently and counts were performed within one hour. Rees-Ecker diluting fluid (Pohle modification)(6) was used. One pipette was used and one chamber counted. All samples were counted by one investigator experienced in platelet counting. These counts were found to be accurate to within 20,000 platelets (coefficient of variation of 10%) for a single count. Since it was found that there is some variation in the normal platelet counts in different individuals, changes in counts were expressed in per cent of the base line count for the individual tested. Eosinophils were counted from the same sample in a hemacytometer counting chamber after dilution with the phyloxine-ethylene glycol diluent described by Roche, Thorn and Hills(8) and by Henneman, Wexler and Westenhaver(2). It was found that 50 mg or more of ACTH (Armour) intramuscularly regularly elicited a maximal "stress" effect as evidenced by the eosinophil response. Accordingly 50 to 100 mg ACTH was administered intramuscularly in 17 individuals after determining the platelet and eosinophil counts before injection. Two others received intravenous ACTH and one who had previously undergone splenectomy received intramuscular ACTH.

Results. As may be seen from Fig. 1, the subjects developed the expected eosinopenia induced by ACTH and also a concurrent, though more transitory, platelet decrease. The thrombocytopenia is more precipitous but less sustained than the eosinopenia. Twenty-four hours after injection both eosinophils and platelets had returned to pretest levels. The decrease in platelets, amounting to an average of almost 50% at its greatest point, is statistically significant.

The individual without a spleen who received ACTH showed changes similar to the 17 normals. The two individuals who received ACTH by the intravenous route over an 8-hour period had a slightly slower but a more sustained decrease in platelets than the 18 who received it intramuscularly.

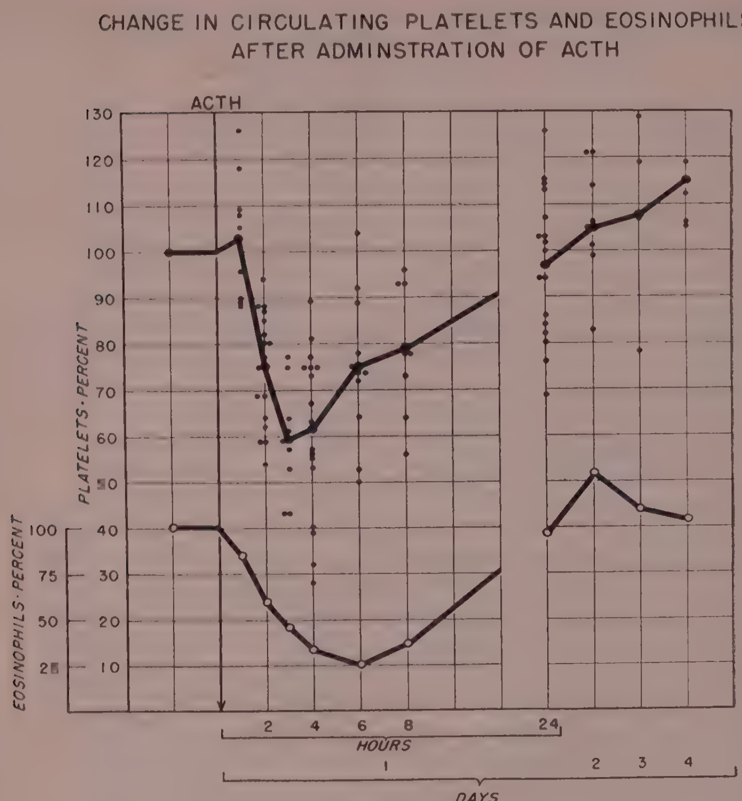

FIG. 1.

Conclusions. 1. After administration of ACTH in doses of 50 to 100 mg to 19 normal individuals there was a temporary significant decrease of platelets in the peripheral blood. This decrease was slightly more rapid and less sustained than the coexistent eosinophil decrease. 2. A similar thrombocytopenia was observed following administration of ACTH to an individual with no spleen.

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Effect of Bromobenzene and Cystine Administration On Vitamin A Deficient Rats.* (19494)

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Outside of its demonstrated role in the mechanism of vision, the biochemical function of vit. A is still unknown. Meunier, Ferrando and Perrot-Thomas(1) investigated the possibility that vit. A might be directly implicated in the synthesis of cystine. In order to investigate this possibility, they made use of the fact that mammalian organisms can be depleted of cystine by administration of bromobenzene; this substance conjugates with cystine to form bromophenyl mercapturic acid, which is then excreted in the urine(2,3). It had been shown previously by Haley and Samuelson(4) that adding 2% bromobenzene to an otherwise normal diet resulted in a decrease of the vit. A content of the liver of the rats of the order of 60% after 3 months. Meunier and his co-workers used 5 groups each of 4 or 5 animals fed a basal, vit. A-free diet containing 15% casein. The diet of one group a) was left unsupplemented. The diet of the other 4 groups was supplemented as follows: b) 1% bromobenzene, c) 1% bromobenzene and 0.12% cystine, d) 2.5 μg of vit. A per animal per day, and e) 1% bromobenzene and 20 μg vit. A per day. No differences were found between groups b and c as regards weight loss or time of survival (average survival time for both groups, 25 days). Groups d and e survived normally and showed average weight gains of 4.9 and 6.9 g per week respectively. Survival in group a (unsupplemented) was 33 days. Meunier and his associates concluded that vit. A was specifically responsible for the synthesis of cystine. However, it must be noted that the level in the diet of the sulfur-containing amino acid corresponding to 15% casein(5) was apparently sufficient to nullify the effect of the cystine supplement in the case of groups b and c, and that their other groups lacked proper controls. No record of the food intake was mentioned.

High levels of dietary protein seem to have a sparing effect on vit. A deficiency(6); epithelial tissues, which are particularly sensitive to vit. A deficiency, show special affinity for administered radioactive cystine(7); it appeared, therefore, useful to study again the possible interrelationship of vit. A and cystine metabolism in bromobenzene intoxication. It seemed important, in this study, to limit the cystine content of the diet to the lowest level compatible with some growth, to increase the bromobenzene effect by increasing its concentration in the diet to the highest level compatible with absence of acute toxic effects and to keep records of the food intake of the different groups.

Experimental. Forty-eight weanling (21-day-old) male rats, weighing on the average 50 g and obtained from the Charles River Stock, were divided into 8 groups of 6 animals. They were fed the following basal diet: casein (Labco), 9%; sucrose, 82%; corn oil, 5%; salt IV, 4%; and choline, 0.1%. The following vitamin supplements were added per kg of ration: thiamine, 10 mg; riboflavin, 10 mg; pyridoxine, 10 mg; nicotinamide, 20 mg; calcium pantothenate, 20 mg; folic acid, 1 mg; biotin, 0.2 mg; para-aminobenzoic acid, 100 mg; inositol, 100 mg; menadione, 15 mg; and alpha tocopherol, 40 mg. Vit. A, when given, was administered every week in doses corresponding to 400 I. U. per day. Bromobenzene (A. R.) when given was added to the diet daily (in solution in the corn oil) at a concentration of 0.5% during the first 10 days and 2% after that time to the end of the experiment; cystine, when given, was incorporated in the diet at a concentration of 0.2%. The groups received the following supplements: vit. A: groups A, AC, AB, ABC; bromobenzene: groups B, BC, AB, ABC; cystine: groups C, AC, BC, ABC; group O received no

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TABLE I. Wt Gains and Food Intake of Non-Deficient and Vit. A-Deficient Rats Receiving Bromobenzene and Cystine.

Groups	Treatment	Wt gain at 40th day, g	Avg food intake (33rd-38th day), g per day
AC	Vit. A, cystine	116.3 ± 18	13.8
A	Vit. A	58.8 ± 22.9	12
ABC	Vit. A, cystine, bromobenzene	80 ± 16.8	10.8
AB	Vit. A, bromobenzene	32.8 ± 3.6	8
C	Cystine	64.7 ± 23.9* (60 ± 21)	9.4
O	—	36.6 ± 17.6	9.7
BC	Bromobenzene, cystine	54.5 ± 18.6* (53.3 ± 22.4)	9.9
B	Bromobenzene	21.4 ± 5.5	7.9

* Figure in the column in this case is the max wt gain; the figure in parenthesis is the wt gain at 40 days.

Stand. dev. calculated by the formula $\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$.

Student's t computed for comparison between groups by formula $t = \frac{(M_1 - M_2)\sqrt{N_1 + n_2 - 2}}{\sqrt{\Sigma d_1^2 + \Sigma d_2^2}}$.

supplement. All animals were kept in individual screen-bottomed cages at constant temperature and under regular conditions of illumination. Food intake was recorded every day and weight every two days.

Results and discussion. Essential results, concerning maximum weight gains of the deficient animals, rates of weight gain of the non-deficient animals and average food intake are recorded in Table I. Groups O and B reached their maximum weight gain 40 days after the start of the experiment; group BC after 39 days; group C after 35 days. Averages and standard deviations of the weight gains of all groups are therefore given at the 40th day for all groups. In the case of groups BC and C, maximum weight gains are also given.

All deficient animals lost weight and declined rapidly after the 40th day and showed typical signs of vit. A deficiency. The severity of these symptoms was in close relation to previous rates of growth.

It is apparent from consideration of the weight gains of non-vit. A deficient animals, that, at the level of protein used, cystine was the limiting factor in growth. Weight gains of animals receiving cystine, (AC, ABC) were twice as great as those of corresponding animals (A, AB) whose diet was left unsupplemented. ($p = 0.002$ and $p = 0.001$ respectively). Administration of bromobenzene caused definite limitation of weight gain, in a manner compatible with its known effect on cystine metabolism. Groups ABC and groups AB showed a weight gain very significantly ($p = 0.002$) smaller than that of AC and A respectively; the addition of bromobenzene to the diet supplemented with cystine caused a reduction in weight gain of the order of 31% and, in the case of the diet not supplemented with cystine, of 44%. The supplement of 0.2 cystine would permit a theoretical maximum of 16% of the bromobenzene to be detoxified as bromophenyl mercapturic acid (2). The fact that group ABC grew significantly ($p = 0.05$) better than group A, seems to indicate that all the cystine supplement was not conjugated with bromobenzene.

In the deficient group cystine was again the limiting factor as regards maximum weight reached. (Difference between C and O, $p < 0.02$; difference between BC and B, $p = 0.002$). The depressing effect of bromobenzene on growth was definite, particularly in the case of the animals receiving no cystine. (Difference between O and B, $p = 0.002$). Decreases due to administration of bromobenzene were somewhat smaller than in the case of the animals receiving vit. A, doubtless because of smaller over-all growth and resulting smaller cystine requirements.

The food intake values reflected observation on growth: cystine increased food intake (AC, ABC, as compared to A, AB), bromobenzene depressed it (A, AC as compared to AB, ABC). The differences between deficient groups (C and O, for example) were masked by the fact that C showed deficiency signs and anorexia before O; ABC, although it grew more than A, did present some periods of anorexia and arrest in weight gains which

were compensated for in the following periods. The values given in Table I correspond to the 15 days comprised between the 25th and the 38th day of the experiment, before any group showed a decline in food intake. Such a decline started on the 38th day for group C, on the 40th day for other deficient groups.

These results do not support the hypothesis that vit. A intervenes directly in the synthesis of cystine, as claimed by Meunier and his associates. For one thing, while the inclusion of cystine in the diet did increase the maximum weight gain of the animals fed vit. A deficient diets, it did not delay the onset of the deficiency. As a matter of fact, weight loss occurred earlier in group C and symptoms were more acute among the animals of this group. Secondly, adding bromobenzene to the vit. A-deficient diets did not cause a more drastic effect than in non-vit. A-deficient animals. If vit. A was specifically instrumental in the synthesis of cystine, one would expect the vit. A-deficient animals, whose capacity of cystine synthesis should already be impaired, to be more immediately and more severely affected by the bromobenzene.

Summary. A study of the effect of bromobenzene administration to vit. A-deficient animals on low-protein diets, supplemented or not supplemented with cystine, and to their controls, does not support the theory, postulated by some authors, that vit. A is directly implicated in the synthesis of cystine.

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Manometric and Histochemical Demonstration of Tyrosinase in Foetal Guinea-Pig Skin. (19495)

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At least some of the skin and hair pigments of mammals (melanins) appear to be produced from the precursor, tyrosine, and it has recently become possible to demonstrate tyrosinase activity in normal mammalian skin. A histochemical demonstration of melanin formation from tyrosine was made in the case of non-pigmented human skin subjected to erythema doses of ultra-violet irradiation(1), and tyrosinase activity has also been demonstrated in skin homogenates from mice and foetal guinea pigs by means of manometric measurements of oxygen consumption(2-4). Since this pigment-forming process has been demonstrated either histochemically or manometrically, it was considered desirable to attempt to demonstrate both aspects of the process in the same sample of tissue.

Methods. In the experiment reported here the intense brown portion from the back of an approximately 52-day-old brown-and-white-spotted foetal guinea pig was separated from the white portion, placed in a frozen mortar surrounded by dry ice, ground to a frozen powder by means of a previously frozen pestle, and this frozen powder was subsequently suspended in pyrex-redistilled water. Four Warburg reaction vessels, each with a volume of approximately 10 ml, were used. Each vessel contained 0.2 ml of 20% KOH in the center

*Public Health Service Research Fellow of the National Cancer Institute, with additional support by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

well, as well as 1 ml of skin suspension in the main portion of the vessel. Each sidearm of two of the vessels contained merely 0.5 ml of M/10 phosphate buffer, pH 6.8 (control vessels), while each sidearm of the other vessels contained 0.5 ml of L-tyrosine dissolved in the same buffer, at a concentration of 0.5 mg/ml (experimental vessels). After 10 minutes of temperature equilibration in a water bath at 38°C, the sidearm contents were tipped into the main portions of the vessels (zero time) and oxygen consumption measurements were obtained. The *net oxygen consumption*, attributable to the enzymatic oxidation of tyrosine, was obtained by subtracting the mean endogenous oxygen consumption of the controls from the mean oxygen consumption in the experimental vessels. Immediately after the Warburg run, skin fragments from one control vessel and from one experimental vessel were removed and fixed in 1:10 formol-alcohol (approximately 15 minutes), dehydrated in absolute ethyl alcohol (approximately ½ hour), cleared in benzene (overnight) and mounted in Permount (Fisher Scientific Co.). Photomicrographs of the experimental and control skin fragments were made under low-power magnification under as equivalent conditions of plate exposure as possible; the two sets of fragments being mounted on the same slide, thus necessitating only a change of fields for the 2 different photographs.

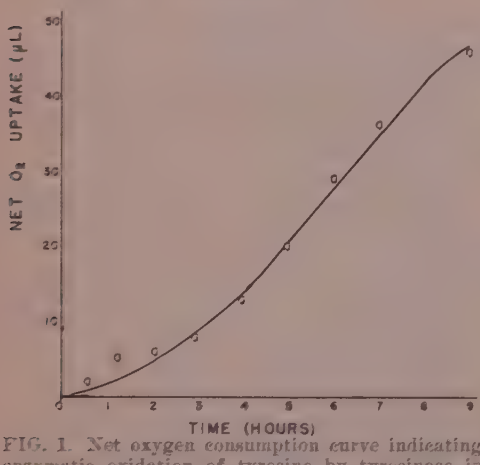

FIG. 1. Net oxygen consumption curve indicating enzymatic oxidation of tyrosine by tyrosinase in brown skin (see text).


FIG. 2. Whole mount of brown skin fragment in the absence of tyrosine (controls). Note the relatively uniform pigmentary details extending from the hairs down to the hair bulbs.

Results. The curve for the net oxygen consumption, indicating the enzymatic oxidation of tyrosine, is shown in Fig. 1. (Other tests (4) have demonstrated specificity of the skin catalyst for the L-configuration of tyrosine, poisoning by phenylthiourea, and enhancement of net oxygen consumption and pigment-production by iodoacetamide.) Correlated with this oxygen consumption was the formation of a brown-black pigment suspension in the experimental vessels, as well as a marked darkening of the larger skin fragments in these vessels. Histological comparison of skin fragments from experimental and control vessels revealed a marked difference in the intensity of hair bulb pigmentation. The control hair bulbs contained brown pigment, but were not completely filled with this pigment. The experimental hair bulbs, on the other hand, appeared to be almost choked with very dark brown pigment. Moreover, this intense pigmentation extended for some length distal to the hair bulb. These differences are indicated in the photographs shown in Fig. 2 (control) and 3 (experimental).

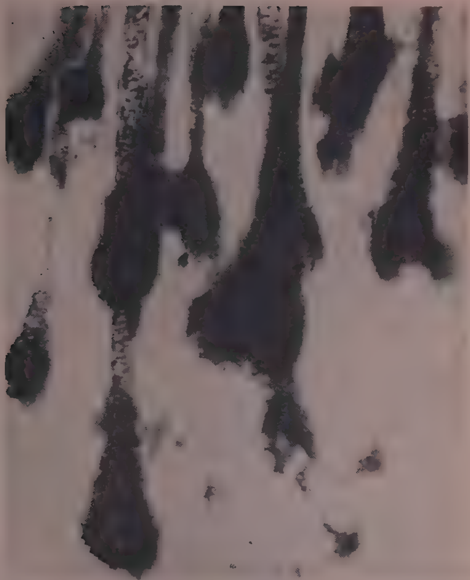

FIG. 3. Whole mount of brown skin fragment in the presence of tyrosine (experimentals). Note the very densely pigmented hair bulbs and lower hair portions. Note also upper hair portions, showing the same pigmentary details as found in the control hairs.

Thus, presumably for the first time, are demonstrated both the enzymatic oxidation of, and pigment-formation from, tyrosine, in the same sample of normal mammalian skin.

Discussion. The positive results reported here strongly indicate that at least some of the dark mammalian hair and skin pigments are products of the tyrosinase system. Studies on a rather extensive series of guinea-pig coat-color genotypes fail, however, to reveal any simple parallelism between tyrosinase or dopa oxidase activity and genetically determined amount or kind of natural pigment(4). These studies, which will be reported and considered in detail elsewhere, confirm in certain major respects previous studies of dopa reactions in the case of guinea-pig skin(5,6).

I am indebted to Professor Sewall Wright for suggestions and criticisms and providing genetic material, bred and identified by him; to Professor Hewson H. Swift, for help in histological technic and photomicrography; and to Professors Benson Ginsburg and Dorothea S. Miller, for laboratory facilities.

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Cultivation of Lansing Poliomyelitis Virus in Tissue Culture. II. Utilization of Glucose in Synthetic Medium.* (19496)

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(Introduced by R. C. Parker.)

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The work of Enders and his associates (1-3) has indicated that poliomyelitis virus can be propagated in cultures of human embryonic tissues. In their work, Hanks-Simms' fluid was used as the source of nutrient. Recently, we have shown(4) that this fluid can be replaced by a synthetic nutrient medium, mixture No. 199, devised by Morgan, Morton, and Parker(5,6). Mixture 199 is of chemically defined composition, and allows of survival of suspended tissue fragments and proliferation of virus for a prolonged period. Hitherto, metabolism has been followed by observing changes in pH. Enders, for example, has reported that the pH values of uninfected cultures are lower than those of infected cultures. Since the Meyerhof sequence of glycolysis has been found to apply to almost all biologic systems, it seemed appropriate to

* Aided by a Grant from The National Foundation for Infantile Paralysis.

study the utilization of glucose in infected and uninfected cultures, in the belief that the state of metabolism could be determined with a high degree of sensitivity.

Methods. Three experiments on the metabolism of tissue cultures infected with Lansing virus have been carried out so far. In each experiment, brain and cord was obtained from a different human embryo. The technic was that described by Weller and Enders(7), and used in our previous study (4). The tissue was minced and distributed between 25 ml Erlenmeyer flasks (8 for each series); mixture 199 was used as the source of nutrient. Hanks-Simms' medium was also used in Exp. 1 for comparative purposes. In Exp. 1 and 3, the source of virus was 0.1 ml of a 10% suspension of mouse central nervous system infected with the Lansing strain of poliomyelitis virus. In Exp. 2, the virus inoculum consisted of 0.1 ml of the 14th culture fluid removed in Exp. 1. In the 3 experiments, virus suspension was added to 4 of the Erlenmeyer flasks, leaving the remaining 4 to serve as uninoculated controls. The culture fluid was removed and replaced with fresh medium every 3-5 days, or when the pH fell to 6.8. Mouse infectivity tests were carried out by the cerebral inoculation of 0.03 ml of pooled culture fluids. Glucose estimations were carried out on pooled uninfected and pooled infected culture fluids in Exp. 1 and 2, and on individual culture fluids in Exp. 3. Glucose estimations were carried out according to the method of Nelson(8). The glucose level of the media used was about 100 mg%. The results are expressed in terms of % glucose utilization.

Results. Exp. 1. The results are shown in Table I. It will be seen that glucose was utilized by the tissues in the Hanks-Simms' medium for 29 days, but in the 199 synthetic mixture series it was still being utilized, although at a low level, when the experiment was terminated at 78 days. It will also be noted that glucose utilization was lower in both series of infected cultures than in the control uninfected cultures. Infectivity tests in the Hanks-Simms' series showed that there were only isolated deaths among mice inoculated with culture fluids later than the 7th. However, in the 199 series, deaths occurred in mice inoculated with all culture fluids up to and including the 16th.

TABLE I. Glucose Utilization and Infectivity Tests in Cultures of Human Embryonic Brain and Cord Infected with Lansing Poliomyelitis Virus.*

Culture fluid	Incubation, days	% glucose used: Uninfected	% glucose used: Infected	Mouse† deaths
		Hanks-Simms' medium		
1	2	54.2	53.4	5/6
2	6	48.6	42.6	2/6
3	10	43.4	33.3	6/6
4	13	31.4	21.9	5/6
5	17	23.6	9.4	3/6
6	21	4	.0	4/6
7	25	15.7	3	3/6
8	29	8.8	2.2	1/6
9	33	.0	.0	1/6
10	38	.0	.0	0/6
11	42	.0	.0	1/6
12	46	.0	.0	0/6
13	50	.0	.0	0/6
14	54	.0	.0	0/6
15	59	.0	.0	0/6
		Synthetic mixture 199		
1	5			6/6
2	9	56.3	47.3	5/6
3	13	58	31.8	6/6
4	16	57.5	29.4	6/6
5	20	62	29.9	5/6
6	23	44	28	5/6
7	27	37.6	21.7	2/6
8	32	47.7	28.2	6/6
9	36	29.1	13	5/6
10	40	29.5	14.8	6/6
11	44	5.4	.0	6/6
12	48	22.7	14.3	4/6
13	53	8.5	1.9	3/6
14	57	8.5	2.9	5/6
15	61	7.3	2.3	6/6
16	65	8.7	3.1	3/6
17	69	5.9	.0	0/6
18	74	11.5	7.1	0/6
19	78	5.2	.0	0/6

* Cultures inoculated with 500 LD_{50} (for mice) of Lansing virus mouse pool.

† Mice inoculated cerebrally with .03 ml of pooled infected culture fluids.

Exp. 2. The results are shown in Table II. It can be seen that the amount of glucose used is similar in infected and uninfected cultures for the first 3 pairs of culture fluids; the characteristic differences appeared subsequently. It was noted that from about the 20th day the infected tissue fragments underwent marked degeneration, resulting in a very turbid medium. This would appear to be due to the cytopathogenic effect(3).

TABLE II. Glucose Utilization and Infectivity Tests in a First Subculture of Lansing Virus in Human Embryonic Brain and Cord, with Synthetic Mixture 199.*

Culture fluid	Incubation, days	% glucose used: Uninfected	% glucose used: Infected	Mouse deaths
1	3	94.8	96.6	1/6
2	7	76.4	84.8	5/6
3	11	87.6	92.8	6/6
4	15	90.4	44.7	6/6
5	19	74.8	20.3	6/6
6	22	55.3	12.8	6/6
7	26	56.7	9	5/6
8	29	35.2	6.1	0/6
9	33	30.5	6.5	0/6
10	36	20.2	3.9	0/6
11	40	13	5	0/6

* Cultures inoculated with .1 ml of the 14th culture fluid of Exp. 1 (Mixture 199).

Exp. 3. The results are shown in Table III. It will be seen that during the first 3 days the amount of glucose used by the tissues is about the same in infected and uninfected cultures. Thereafter, the values for glucose utilization fell more rapidly in the infected than in the uninfected cultures. Thus, the amount of glucose used in these cultures followed the same general pattern as that seen in the previous experiments.

Discussion. It has been shown, in confirmation of our previous work, that the Lansing poliomyelitis virus proliferates in cultures of human embryonic brain and cord supplied with synthetic nutrient mixture 199, devised by Morgan, Morton and Parker(5). Tests for glucose utilization in culture fluids have shown that these values are significantly lower in cultures containing virus than in the uninfected control cultures. The apparent indications are that when glucose utilization falls to a low level, the liberation of virus into the culture fluids, as shown by infectivity for mice, also falls. It is suggested that tests for glucose utilization are of value in studies of the growth of Lansing virus in tissue culture, as they give an indication of the metabolic state of the tissue in which the virus grows. In this connection, it is of interest to recall that Racker and Krimsky(9) showed an inhibition of glucose utilization in homogenates of brains of mice infected with Lansing virus.

Ackermann has postulated that virus is formed in infected tissue due to an abnormal sequence of some normal metabolic cellular process, and that the functioning of the Krebs cycle is essential for the propagation of influenza virus(10). He also found that dl-ethionine and dl-methionine prevented the propagation of influenza PR8 virus and Lansing poliomyelitis virus, and that this inhibition could be overcome by addition of l-methionine(11,12).

It appears possible that the proliferation of Lansing virus in fragments of human embryonic tissues can be studied when these tissues are suspended in a synthetic nutrient medium, and that metabolic studies may be made by addition or removal of the component parts

TABLE III. Glucose Utilization and Infectivity Tests in Cultures of Human Embryonic Brain and Cord Infected with Lansing Virus, with Synthetic Mixture 199.*

Culture fluid	Incubation, days	% glucose used: Uninfected				% glucose used: Infected				Mouse deaths
1	3	86.8	95.5	65.3	91.4	87.2	91.7	86.2	90.9	6/6
2	7	61.4	36.1	43.5	58.2	43.6	52.2	47.4	46.2	6/6
3	11	62.7	53.8	32.4	49.5	21.1	31.2	35.9	28.1	5/6
4	15	49.7	48.2	30.2	50.5	14.1	20.7	16	17.5	4/6
5	19	46.1	41.7	29.2	46	9.3	18.7	14	9.3	5/6
6	24	51.5	46.5	26.7	48.5	7.9	18.9	13.4	4.5	3/6
7	29	35.8	25.7	21.8	37.6	.0	8	1.5	.7	3/6
8	32	28.2	16.7	13.4	33.7	.0	9.3	2.3	2.3	4/6
9	37	41.7	20.7	31.7	50.7	3.8	18.4	14.2	5.1	0/6
10	42	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
11	47	22.8	3.6	10	33.8	.0	4.4	.6	.0	4/6
12	51	15.8	6.7	10.1	23.6	2.3	8.7	6.7	1.5	4/6
13	55	13.4	6.5	5.8	19.4	4.6	8.7	3.1	3.1	2/6
14	58	8	2.2	.8	8.7	.0	.8	.0	1.5	1/6
15	62	3.4	.0	.0	9.1	.0	.4	.0	.4	1/6

* Cultures inoculated with 500 LD_{50} (for mice) of Lansing virus mouse [illegible]

of this medium.

Summary. 1. Lansing poliomyelitis virus proliferates in cultures of human embryonic brain and cord supplied with a synthetic nutrient medium. 2. The amount of glucose used by cultures that contain virus is significantly less than that used by uninoculated control cultures.

The human embryos used in these studies were obtained through the courtesy of the gynecological staff of the Toronto General Hospital. We are indebted to Dr. R. C. Parker and his colleagues for supplies of the synthetic medium.

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Cultivation of Coxsackie Virus in Embryonated Eggs and in Chick Tissue Cultures. (19497)

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First attempts to grow Coxsackie virus in embryonated hens' eggs were unsuccessful(1). Since then there has been only one report of adaptation to growth in eggs. Huebner(2), after alternate mouse to egg passage, carried a Group A, Type 2 strain through a number of consecutive yolk-sac passages. Similarly, there has been a report of successful cultivation in tissue culture. Slater and Syverton(3) maintained a Group A, Type 4 strain in embryonic mouse cell medium for 24 passages.

Results of the following investigation confirm the difficulty of growing the Coxsackie viruses in embryonated eggs and suggest that, while Group A, Type 2, may perhaps be more readily adapted than other types, the individual strain appears to be the most important factor.

Methods and materials. Virus strains. Thirty-one strains were used, including representatives of 10 types of Group A and 3 of Group B. In most cases, 2 or more strains of a given type were tested but only one strain each of Group A, Type 6, 7, and 9 and Group B, Type 2. Twelve strains had been received from other laboratories as muscle suspension or mouse brain; the remainder were isolated in this laboratory from human fecal specimens. Stock supplies of the virus were maintained as infected mouse brain in 50% glycerol. Infected leg tissue suspension was the usual material for egg and tissue-culture inoculation, the exceptions being 2 Group B strains, where brain tissue was used as the inoculum.

Embryonated eggs. After preliminary exploration, the following procedure was used as a screening test. Eggs were incubated for 7 days at approximately 37°C; 0.1 ml of 10% virus suspension was inoculated into the yolk sac and incubation continued for 6 days at approximately 35°C before harvesting embryos and a portion of the yolk-sac tissue. The heads, minus the eyes, of 6 to 8 embryos were weighed, ground with sterilized sand, and a 20% suspension prepared, using as a diluent 0.85% saline containing 10% beef infusion broth. The suspensions were centrifuged for

10 minutes at approximately 2500 rpm. Supernatant fluids were stored in a dry ice cabinet until mouse infectivity tests could be performed. Similar suspensions were prepared from pools of legs and wings, from the remainder of the bodies including the viscera, and from the pooled yolk-sac tissue. These suspensions were prepared to determine the localization of the virus but for passage of untested materials, a mixture of equal parts of the 4 suspensions was used. On the assumption that virus might be present only in one suspension, the use of the mixture provided for no more than a 4-fold dilution of the active material. Generally, suspensions of first passage materials were not tested in mice since positive mouse infectivity tests might be ascribed to survival of the inoculum. *Tissue cultures.* The flask culture technic was used throughout. Prior to the adaptation of any of the strains to eggs, tissue culture studies of Group A, Type 1 (No. 48249) had been made using brain or leg tissue from one-day-old mice, with indifferent success. The strain was carried through a number of passages but with steadily declining titer. The first egg-adapted strain was grown without difficulty *in vitro* on embryonic chick tissue. Other strains were then investigated. The fluid phase consisted of one part ox serum ultrafiltrate, 3 parts Simms X7 solution(4) with sufficient penicillin and streptomycin added to give a concentration of 50 units and 50 μg/ml, respectively. Five ml of the fluid were dispensed into each rubber-stoppered 125 ml Erlenmeyer flask. Chick embryos 6 to 8 days of age were minced and washed in Simms solution. Four drops of tissue were used per flask. The initial inoculum was 0.1 ml of 20% suspension of infected mouse legs or brains or a similar preparation of infected chick embryo legs and wings. Cultures were incubated at 36°C for 7 days, plated, and 0.1 ml transferred to a fresh flask of medium. Fluid from the seed culture was centrifuged for 10 minutes at approximately 2500 rpm. The supernate was stored in a dry ice cabinet until mouse infectivity tests could be performed.

Tests for determining presence of virus. Tissue culture fluids and suspensions prepared from infected egg or embryo tissues were tested for virus by injecting into suckling mice 1 to 3 days of age. The approximate mouse infective titer was determined by injecting serial tenfold dilutions of the materials. The specificity and identification of the egg-adapted or tissue culture strains were checked by neutralization tests using immune sera prepared in mice or hamsters.

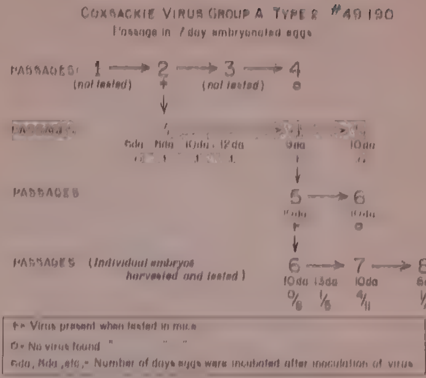

FIG. 1. Coxsackie virus, Group A, Type 2, #49190.

Results. Embryonated eggs. Twenty-four strains representing 10 types of Group A virus and 5 strains which included 3 types of Group B virus were investigated. Negative results were obtained with all Group B strains and with Group A, Types 1, 3, 5, 6, 7, 8, 9, and 10. Only 5 Group A strains gave evidence of virus being present in any quantity in the second passage. One was the Type 4 strain grown by Slater and Syverton(3) in tissue culture; 2 other Type 4 strains gave negative results. The investigation of this strain was not continued. Two were Type 2 strains isolated in this laboratory. Strain No. 49190 was transferred through 8 egg passages with no enhancement of pathogenicity and no gross evidence of infection. Fig. 1 shows the history of these passages. When, with a lengthened incubation period, the strain could not be maintained in passage, it was decided to prepare and test suspensions of individual embryos. Only one embryo of 13 harvested from the 6th passage contained detectable virus; only 4 of 11 were positive in the 7th passage. Since these embryos showed no signs of infection, it was not possible to determine by gross observation which to harvest and use for

passage. The low incidence of infected embryos explains the failure of passages in which pooled tissue of a number of embryos was used. In those embryos that became infected, virus was present in fair titer (10^{-4}). Results with strain No. 50136 were similar. The 2 remaining strains (Nos. 5127 and 50376) were received as Group A, Type 5 from another laboratory. Both gave evidence of marked cross reactivity with Group A, Type 2 and the virus grown in egg and tissue culture was similar serologically to Type 2 only. Strain No. 5127 grew even less readily than those previously described but the Type 2 element of strain No. 50376 immediately adapted to cultivation in eggs and was highly pathogenic for the embryos, occasionally causing death. The infected embryos were pale and swollen, with thin fragile legs and feet curled or bent in abnormal positions. Histopathologic examination of the infected embryos revealed hyaline degeneration of the striated muscles, as is seen in infected mice (1), but no lesions of the central nervous system. This strain was carried through 8 egg passages. A suspension of the pooled legs and wings from embryos of the 7th passage was infective for mice by intraperitoneal injection (0.05 ml dose) when diluted to 10^{-7}. Virus was also present to high titer in other embryonic tissues and to a much less degree in yolk-sac tissue. Three other Type 5 strains failed to grow in eggs.

The results obtained with strain No. 50376 were sufficiently unusual to suggest the presence of another agent. However, Group A, Type 2 antiserum specifically prevented the growth of the strain in eggs. Attempts to increase the pathogenicity for eggs of another Type 2 strain by simultaneous inoculation of Type 5 virus were unsuccessful. Embryo suspensions of No. 50376, made non-infectious for eggs by the addition of Type 2 serum, did not initiate growth when added to egg inoculum of strains of Type 4 and Type 6, previously found not to grow in eggs.

Tissue cultures. Strain No. 50376, adapted to eggs, grew readily in culture on chick embryo tissue. Fluid of the 8th tissue culture passage was infective for mice in a dilution of 1:100,000. Egg passage virus No. 49190, which infected only occasional embryonated eggs, was easily cultivated *in vitro* on embryonic chick tissue. That growth in chick tissue cultures was not dependent on prior adaptation of the virus to eggs was shown by successful cultures obtained with the above strains and strains No. 50136 and No. 5127, using infected mouse leg suspension as inoculum. All attempts to cultivate No. 50376 or No. 49190 in mouse leg or mouse brain tissue culture were unsuccessful.

The Type 4 strain adapted by Slater and Syverton to mouse tissue culture grew also in chick tissue medium. Three other Group A, Type 4 strains did not. Eight additional strains representing Types 1, 3, 5, and 6 failed to grow.

Summary. Among 29 strains of Coxsackie virus tested in embryonated eggs, one strain, serologically Group A, Type 2, exhibited unusual pathogenicity for chick embryos. Of 16 strains tested, the same strain, 3 other Type 2 strains, and a Group A, Type 4 strain grew readily *in vitro* on embryo chick tissue. These results suggest that strain differences are a primary factor in adaptation to growth in embryonated eggs or tissue culture.

The author is indebted to Dr. Hildegard Plager for testing the materials in mice.

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Intravenous Infusions Into Human Subjects of Fractionated Coconut Oil Emulsions. (19498)

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The purification of natural coconut oil is of major importance in the proper development of a fat emulsion for intravenous use in man. Although coconut oil is sufficiently purified for edible purposes, it nevertheless contains a variety of non-essential nonglyceride substances which must be considered as undesirable impurities for inclusion in an intravenous preparation. These latter substances are the hydrocarbons, free fatty acids, phytosterols, carotenoid pigments etc. By means of special filtration and distillation technics natural coconut oil can be refined to yield three fractions of relatively pure fatty acid triglycerides. The present report is devoted to a clinical study in which each of these fractions of coconut oil was emulsified and injected intravenously into human subjects.

Method of Study. Emulsions containing 10% of fraction A or fraction B or fraction C were prepared by homogenization and stabilization with 2% gelatin and 5% glucose in the manner previously described(1). In addition, combinations of fractions A and B, 5% of each, fractions A and C in the same proportion and also fractions B and C were made into emulsions. A total of 24 hospital patients were divided into 6 groups corresponding to the type of emulsion administered. In addition, 8 patients of this series received infusions of all 3 fractions or of their combinations. Each infusion was started in the morning with the patient in the post-absorptive state. During the study period which lasted 24 hours, temperature, pulse, respirations and blood pressure were recorded hourly. Four samples of blood were drawn; an initial sample at the start of the infusion, a sample at the termination of the infusion and samples at 12 and 24 hours. The following determinations were made on the blood; hemoglobin(2), hematocrit cell volume, total lipids (3), and cholesterol(4). Hemolysis *in vivo* was determined by the quantitative estimation of free hemoglobin in plasma by a modification of Flink's method(5). Complete blood counts were also made. Catheterized urine specimens obtained at the same intervals as noted above were examined for ketone products, urinary fat and blood.

Results. Group I. Four patients were each infused with one liter of the emulsion containing 10% fraction A coconut oil. In every case there was a severe reaction during the course of the infusion necessitating its discontinuance. Toleration for this emulsion did not exceed 400 ml. The rate of administration did not appear to alter reactivity. Symptoms were similar in all cases. These were dyspnea, cough with expectoration of frothy sputum, retching and vomiting of gastric contents tinged with blood and considerable bile. The patients complained of severe pain which was localized in the mid-back region corresponding to the site of the kidneys or pancreas. The temperature rose from 4 to 6 degrees even before the infusion was discontinued, persisted for a period of 2 to 3 hours and then subsided rapidly to the initial level. The blood studies showed a significant fall in hemoglobin, hematocrit cell volume and number of red blood cells, particularly in sample 2 and sample 3. Leukocytosis was most marked at the completion of the infusion. The values for total lipids and cholesterol rose above the initial values but declined subsequently to values even below the initial level. Free hemoglobin in plasma was markedly elevated in samples 2, 3, and 4. The intermediate urine samples were positive for acetone. All the urine samples after the infusion were strongly positive for blood.

Group II. Three patients from the previous group and 4 new subjects were infused with the emulsion containing fraction B coconut oil. This emulsion was well tolerated without reaction and with little or no temperature elevation except in 3 subjects who showed a rise which averaged 1.5 degrees in

TABLE I. Data Showing Effects of Each of Three Fractions of Coconut Oil Emulsion Infused into the Same Subject.

Fraction	Sample	Hemoglobin, g %	Hematocrit, Mm	Free hemoglobin, mg %	R. B. C. × million	W. B. C. × thousand	Total lipid, mg %	Cholesterol, mg %	Urine acetone	Urine blood	Temp., °F
A	1	13.5	46	4	4.8	6.5	412	120	0	0	98.6
	2	11.6	40	108	3.2	20.3	680	160	2+	4+	98.6-102
	3	10.8	40	60	3.6	17.1	520	120	2+	4+	104 -100.2
	4	11	41	30	3.8	8.6	480	90	0	2+	100 - 98.6
B	1	14	48	2	5.1	5.8	334	106	0	0	98.6
	2	13.8	47	2	4.8	6	600	122	2+	0	98.6- 99.2
	3	13.8	46	2	4.6	5.6	522	120	0	0	99.2- 98.6
	4	13.9	46	2	4.9	5.3	420	120	0	0	98.6- 98.6
C	1	14.6	50	2	4.6	7.1	572	142	0	0	98.6
	2	14.4	48	2	4.7	7	737	168	2+	0	98.6- 99.4
	3	14	48	2	4.2	6.5	510	118	1+	0	99.6- 98.4
	4	14	49	4	4.4	6.4	430	90	0	0	98.4- 98.8

the last 2 hours of the infusion period. Except for a mild dilution effect, there were no significant changes of any of the constituents of the blood studied in this investigation. Leukocytosis did not occur. Hemoglobinemia was not demonstrable. Urinary findings were within normal limits.

Group III. Four new subjects and the same 3 patients who had been tested with the fraction A and B emulsions received infusions of the emulsion containing fraction C coconut oil. In this group infusion reactions did not occur. Temperature elevations were minimal and did not exceed 1.2 degrees. The laboratory data was similar to that obtained in the Group II studies.

Groups IV and V. The subjects in the latter 2 groups were infused with emulsions which combined fractions A and B or fractions A and C. Reactions were found to occur in every case but were less severe than those encountered when fraction A emulsion alone was administered. The limit of tolerance for either combined emulsion did not exceed 700 ml. Temperature elevations averaged 3 degrees. Leukocytosis was not as great. Free hemoglobin in plasma did not exceed 85 mg %. Clinically, vomiting, cough and back pain were observed as previously.

Group VI. Four patients comprising this group received infusions of the emulsion combining fractions B and C. These infusions were well tolerated without untoward reactions. The laboratory data showed little variation from that noted for Group II or III.

Discussion. The 3 fractions discussed above were prepared by Drs. Barsky and Zinzalian of the research division of the E. F. Drew Co. Each fraction consisted principally of the glyceride esters of a limited number of homologous saturated fatty acids. In the B and C fractions smaller amounts of the esters of the unsaturated fatty acids were also present. Fraction A consisted mainly of a mixture of the glycerides of caprilic and capric acids. Fraction B comprised the esters of lauric and myristic acids and minor concentrations of linoleic and oleic acid esters. Fraction C contained principally the glycerides and palmitic and stearic acids and lesser quantities of the esters of oleic, linoleic, myristic and lauric acids. The concentration of the saturated fatty acid esters in each fraction was 90% or greater. The esters of oleic and linoleic acids which constituted about 6% of the whole coconut oil were unequally distributed in the B and C fractions. As a result of the fractionation processes, non-fatty material and other non-glyceride substances present in natural coconut oil remained as an untested residue.

Emulsions of B and C fractions were well tolerated while serious reactions were found to occur with the intravenous infusions of the emulsions containing the A fraction. The basic patho-physiologic process associated

with the infusion of the reactive fraction appeared to be that of hemoglobinemia due to hemolysis *in vivo*. This can be accounted for on the basis of intolerance of the organism for low molecular weight fatty acids and their esters such as were contained in fraction A. The relative solubility of the latter and their ease of dissociation hastened the process of saponification in the blood and the surface action effect on the red blood cells causing hemolysis and the resultant hemoglobinemia. Better toleration obtained with the injections of the B and C fractions can be accounted for on the relative insolubility of the long chain fatty acid esters retarding in the blood any chemical interaction or hydrolysis.

Summary. 1. Emulsions of the glycerides of the short chain fatty acids were toxic when injected intravenously into human subjects. 2. The glycerides of the high molecular weight fatty acids, lauric, myristic, palmitic and stearic acids were non-toxic when injected in emulsion form. 3. Inclusion with the latter of small concentrations of the fatty acid esters of the type of oleic and linoleic acids did not affect the toxicity of the emulsions.

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α-Lipoic Acid in Oxidative Reactions of a *Corynebacterium*. (19499)

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α-Lipoic acid is a highly active growth factor for several microorganisms(1,2) and functions in the oxidative conversion of pyruvate to acetate by *Streptococcus faecalis*(1). Acetate will replace the factor for the growth of several lactic acid bacteria. On the other hand, acetate will not substitute for a lipoic acid-like material in the growth of a *Corynebacterium*(2,3). This difference prompted the present study of the *Corynebacterium,* since it suggested that α-lipoic acid possesses a function or functions in addition to its role in the formation of acetate from pyruvate.

Methods. The organism,* maintained on yeast extract-glucose-agar slants, was grown in a medium similar to that described by Stokstad, *et al.*(3). Instead of "protogen", a concentrate of lipoic acid was used; this material, potency 50,000 acetate equivalents/mg(4), gave suitably deficient cells at a level of 50 μg/liter or less. To permit aeration of the medium, 1.5 mg/liter of Dow-Corning Antifoam A were added. A 10% inoculum, 24 hours old, was used for 14 liter batches. After 24 hours at 25°C. the cells were harvested with a Sharples Super-Centrifuge, washed with 0.9% saline, suspended in water, lyophilized, and stored at −18°C. The experiments described below were performed with these dried, deficient cells, utilizing the usual manometric methods. The cells and crystalline α-lipoic acid† were mixed just before equilibrating the Warburg flasks.

Lipoic acid effects. A preliminary survey revealed that a variety of substances was oxidized by the dried cells and that the rate of oxygen uptake for many of these was stimulated by α-lipoic acid (Table I, Columns

* Our thanks are due to Dr. E. L. R. Stokstad for a culture of this organism.

† We are indebted to Dr. L. J. Reed for samples of α-lipoic acid.

TABLE I. Effect of Crystalline α-Lipoic Acid on Oxidation of Various Substrates.

	Oxygen uptake (μatoms)			
	Deficient cells		"Aged" cells	
	Without lipoic acid (1)	With lipoic acid (2)	Without lipoic acid (3)	With lipoic acid (4)
Endogenous	14.8	20.9	18.1 9.7*	27.7 5.8*
Substrates: †				
Pyruvate	15.5	21.6	14.6	14
Lactate	18.8	25.9		
l Malate	6.6	20.8	1.6	18.5
Fumarate	7.4	13.8	6.1	23.6
Acetate	9.2	30	2	7.3
α-Ketoglutarate	.8	3.4		
Glutamate	2.2	20.7	.0	11.1
Ornithine	7.5	18.2		
Arginine	45.8	52.6		
Proline	27.7	62.3	8.2 8*	29.2 22.8*
γ-Aminobutyrate	71.1	69	62.4	67.3

* Substrate tipped in after 2 hr. † Oxygen uptakes corrected for endogenous respiration.
Conditions: 7 mg cells and .07 μg α lipoic acid in 1.8 ml .055 M phosphate pH 5.9; .2 ml .2 M substrate tipped in after 15-20′. Duration of exp.: 180′.

TABLE II. Response of "Aged" Cells to Varying Amounts of Crystalline α-Lipoic Acid.

Wt. of α lipoic acid (μg)	Oxygen uptake (μatoms)		
	Endogenous	Proline*	1-Malic acid*
.0	26.2	16.2	1.9
.002	27	22.3	5.9
.01	30.6	37.4	19
.05	33.4	45.5	35.1

* Substrate uptakes corrected for endogenous uptake.
Conditions as in Table I, but exp. was 300 min.

1 and 2). Distinct stimulations were observed with as little as 0.002 μg lipoic acid per Warburg flask (Table II). The endogenous activity is high and is stimulated by the growth factor under the conditions used in most experiments; however, it should be noted that low lipoic acid levels produce substrate stimulations without appreciable effect on the endogenous activity (Table II).

The finding that pyruvate is oxidized by this organism raises the possibility that pyruvate oxidation is the rate-limiting step in the oxidation of the other substrates. Since α-lipoic acid functions in pyruvate oxidation (1) and shows a stimulatory effect with pyruvate here, the possibility then exists that the stimulatory effects observed with the other substrates are the result of a single function. However, the results described below appear to rule out this explanation.

Deficient "aged" cells. Table I, Columns 3 and 4, presents results of experiments performed with a batch of cells which had been in use over a period of 3 months. These "aged" cells could still oxidize pyruvate, but the rate of oxygen uptake was no longer stimulated by α-lipoic acid. Nevertheless, the stimulatory effect on the other substrates is still in evidence. This finding suggests a role for lipoic acid in the oxidation of these substances which is distinct from its known role in pyruvate oxidation. Attention is drawn to the observation that preliminary aeration of the cell suspension reduces the blank activities considerably without greatly affecting the stimulatory effect of lipoic acid on proline oxidation (starred values, Table I). In fact, the stimulation was observed with cells in which the endogenous activity was now *lower* in the presence of lipoic acid (because of greater depletion of endogenous substrates during the aeration).

Effect of high pH. All experiments described above were performed at pH 5.9. At pH 9.2, with fresh cells, pyruvate oxidation was reduced to a very low level, yet proline oxidation was considerable and showed stimulation by lipoic acid (Table III, Columns 1 and 2). The activity of the blanks at this pH was of minor proportions. The pyruvate

TABLE III. Effect of α-Lipoic Acid on the Oxidation of Various Substrates at High pH.

Substrate	Oxygen uptake (μatoms) Deficient cells: Without lipoic acid (1)	Deficient cells: With lipoic acid (2)	"Aged" cells: Without lipoic acid (3)	"Aged" cells: With lipoic acid (4)
Pyruvate	.3	2	.0	.0
Proline	11.1	23.3	2.8	11.2
γ-Aminobutyrate			3.1	8.7
None	2.8	5.9	5.7	9.2

Conditions: 7 mg dry cells, .07 μg α-lipoic acid, and 50 μmoles K_2HPO_4 in 1.8 ml tris(hydroxymethyl)aminomethane buffer, .11 M. .2 ml .2 M substrate tipped in after equilibration. Columns 1, 2: pH 9.2; duration 120′. Columns 3, 4: pH 8.8; duration 150′. Oxygen uptake corrected for endogenous respiration.

activity appears to be much too small to account for the lipoic acid effect on proline. With "aged" cells at pH 8.8, there is no pyruvate oxidation at all, yet proline and γ-aminobutyric acid are oxidized and respond to the growth factor (Table III, Columns 3 and 4). At this pH, acetate, malate, and glutamate are also not attacked, and it may be concluded that lipoic acid operates in proline and aminobutyrate oxidation at a point distinct from its function in the oxidation at the lower pH of the other substrates mentioned here.

Non-accumulation of Pyruvate. If in this organism α-lipoic acid were involved only in pyruvic acid oxidation, one would expect pyruvate (under the conditions used) to *accumulate* during substrate oxidation in the absence of the factor. Analysis of the reaction mixtures(5) revealed no accumulation of pyruvate as a result of acetate, proline, or endogenous oxidation, with or without added lipoic acid. This supports the previous evidence and indicates a non-pyruvate function for lipoic acid.

Glutamate oxidation. A trapping agent, 2,4-dinitrophenylhydrazine, was used to seek carbonyl products of glutamate oxidation. The reagent was incubated with dried deficient cells at pH 5.9 buffer, with and without sodium glutamate, and with and without α-lipoic acid. The cells were centrifuged off and the supernatants acidified and extracted with ethyl acetate. The organic extracts were analyzed by paper chromatography, using two different solvents(6). Spots corresponding in position to pyruvate and α-ketoglutarate were found in all samples. The spots were all equally faint except the ketoglutarate spot obtained from the glutamate-lipoic acid combination; this was markedly more intense. This agrees with the manometric data and suggests that formation of ketoglutaric acid from glutamic acid is stimulated by lipoic acid.

γ-Aminobutyric acid oxidation. Since the metabolism of this compound has been studied so little, and because the *Corynebacterium* shows such a high rate of oxidation, further studies were made of the mode of oxidation. A trapping experiment like the one above resulted in pyruvate and ketoglutarate spots which were more intense than the controls, the pyruvate considerably so. The pyruvate spot was identified further by elution with pH 7.4 phosphate and examination with a spectrophotometer. In agreement with a sample of pyruvate similarly treated, an absorption peak was found at 370 mμ; addition of alkali moved this to 445 mμ. Succinaldehydic and formylacrylic acids, possible intermediates, were ruled out by comparison of R_F values and absorption spectra. The closely related compounds, γ-hydroxybutyric acid, δ-aminovaleric acid, and β-aminopropionic acid are not attacked.

Other metabolites. Glycolic, glyoxylic, citric, itaconic, and succinic acids were inert as substrates, or nearly so; similarly, with glycine, alanine, leucine, phenylalanine, lysine, and hydroxyproline. Formate was oxidized rapidly, but without influence by α-lipoic acid.

Putrescine was the most active substrate tested; oxygen uptake by deficient cells proceeded rapidly until 5 atoms/mole putrescine were taken up. The uptake then equaled that of the control. α-Lipoic acid did not speed

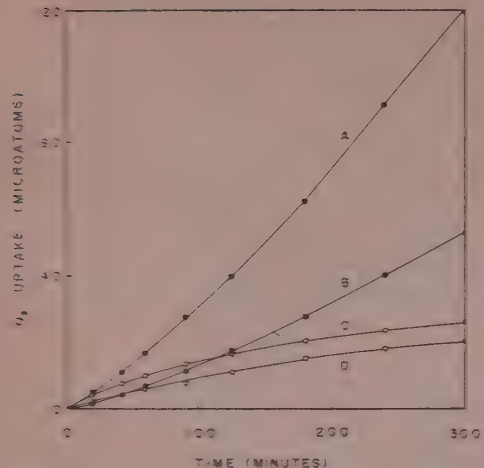

FIG. 1. Effect of α-lipoic acid on the oxidation of proline by deficient cells. Conditions as in Table I. Curves A and B represent response to proline as the substrate in the presence (A) and absence (B) of α-lipoic acid. Oxygen uptake values are corrected for endogenous respiration, represented by curves C (α-lipoic acid present) and D (α-lipoic acid absent).

the oxidation, but prolonged it so that ultimately the ratio was 6 instead of 5. The number of atoms required for complete oxidation is 11. As would be expected from these ratios, very little carbon dioxide was produced: about 1.5 moles/mole putrescine, with or without lipoic acid. Cadaverine was not attacked. *Anaerobically*, very little carbon dioxide is produced by the cells. Pyruvate yields a trifle, but glutamate, proline, and ornithine are inert.

Discussion. The high blanks found in most experiments and the complexity of the enzyme system used, of necessity, make tentative the conclusion that lipoic acid has multiple functions. However, it has been shown that the endogenous activity appears to be independent of the qualitative effects of lipoic acid on various substrates. Moreover, extension of the experiment duration produces a lower ratio of blank:substrate activity without affecting the qualitative results, as illustrated in Fig. 1. Furthermore, in practically all experiments α-lipoic acid revealed its stimulatory effect within the first 10 minutes after tipping in the substrate. The rapid effect appears also when there is no pre-incubation of the growth factor with the cells, as found in comparisons with acetate, malate, and proline. This, and the use of dried cells, would appear to rule out some indirect effect, such as adaptive formation of enzymes.

Summary. The metabolism of dried cells of a *Corynebacterium* has been studied and evidence presented that indicates α-lipoic acid is involved in the oxidation of a variety of substrates and that the locus of action is at several independent sites.

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